Comparison of physiological and anatomical changes of C3 (*Oryza sativa* [L.]) and C4 (*Echinochloa crusgalli* [L.]) leaves in response to drought stress

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Abstract. The experiment aimed to analyse the different response of C3 (*Oryza sativa* L.) and C4 (*Echinochloa crusgalli* L.) species to drought stress based on physiological and anatomical properties. Seeds of rice (*Oryza sativa*) and Echinochloa (*Echinochloa crusgalli*) were grown in 15 cm (D) pot for 6 weeks under well-watered conditions. After 6 weeks the plants were divided into two groups, (1) well-watered which were watered daily, and (2) drought stress which were withheld from watering for 6 days. After 6 days of drought, the plants were then re-watered to analyse plant recovery. During drought period, the plants were analysed for growth, leaf relative water content (RWC), photosynthesis, and leaf anatomy. Drought stress significantly reduced leaf RWC of both species, but the reduction was bigger in rice than in Echinochloa. The maximum efficiency of photosynthesis (Fv/Fm) was decrease significantly in response to drought stress by about 48.04% in rice, while it was only 34.40% in Echinochloa. Anatomical analysis showed drought treatment tended to reduce leaf thickness in the area of bulliform cell, major- as well as intervein and xylem diameter, more in Echinochloa than in rice, suggesting that the decrease of vein and xylem diameter is among the anatomical parameters that is important to overcome from drought stress in Echinochloa. The number of chloroplast in the mesophyll cell and bundle sheath cell (BSC) was different between these two species, where in Echinochloa chloroplast was found in both mesophyll as well as BSC, while in rice it was only found in mesophyll cell, confirmed that Echinochloa is a C4 and rice is a C3 species. Interestingly, in Echinochloa, the number of chloroplast was significantly increased due to drought stress in BSC, but not in mesophyll cell. The number of starch granules also dramatically increased in response to drought in the mesophyll cells of rice and Echinochloa, and in the bundle sheath cell of Echinochloa which indicate that C3 cycle may be occurred in C4 species, at least in Echinochloa, especially during drought stress.

Keywords: C3 and C4 plant, drought stress, *Echinichloa crusgallii*, rice, photosynthesis, leaf anatomy, starch, chloroplast.

1. Introduction

Under natural conditions, plants usually undergo environmental stress due to lower precipitation and soil water content that is known as "drought stress". Drought stress or water stress may arise in several ways including high rate of evaporation, the existence of osmotically active compounds, and inadequate water uptake due to shallow soil[1]. Drought is a major abiotic stress which causes detrimental effect on most plant growth stages and decreases crop yield, and therefore being a severe threat to sustainable agriculture[2]. Water stress, due to limitation of water supply from root or because of higher transpiration rate, severely impair growth and development of plants. Even at moderate water deficit, the rate of CO_2 assimilation in the leaves is depressed, mostly due to stomatal closure[3,4].

The effect of water stress to plant depends on the plant genetic and the degree of the stress. The difference of plant morphology, anatomy and metabolism may result in variations of response to water deficit. Due to differences in metabolism, there are some differences between C3 and C4 plants in response to water stress. In C3 species, drought stress is normally followed by photosynthetic rate reduction due to lowering stomatal conductance which consequently increases photorespiration[5]. On the other hand, C4 species is able to maintain photosynthetic rate under mild drought due to its mechanism known as CO_2 concentrating mechanism (CCM) which enables the plant to suppress photorespiration[6,7]. These two species in many cases have differences in leaf anatomy especially the existence and the role of bundle sheath cell (BSC) in the plant photosynthesis[8]. We believe that this differences may cause variations in response to drought stress indicated by physiological and metabolic processes. Therefore, we compared *Oryza sativa* L. (C3) and *Echinochloa crusgalli* L. (C4) to analyse the different response of these two species to drought treatment in a pot series of experiment.

The objective of this experiment was to analyse the different response of C3 (*O. sativa*) and C4 (*E. crusgalli*) species to drought stress based on physiological and anatomical characteristics.

2. Methods

Seeds of rice (*O. sativa*) and Echinochloa (*E. crusgalli*) were germinated in plastic container for 2 weeks. The seedlings then were transplanted and grown in 15 cm (D) pots. The plants were watered daily, and fertilizer was applied at the planting time and three weeks after planting using NPK (12-12-12) fertilizer of 12.5 gram per pot. After 6 weeks the plants were divided into two groups, (1) well-watered which were supplied with water daily, and (2) drought stress which were withheld from watering for 6 days. After 6 days drought treatment, the plants were then re-watered to analyse plant recovery.

During the period, the plants were analysed for growth, leaf relative water content (RWC), photosynthesis, and several leaf anatomy parameters. Leaf RWC was analysed using 10 circular leaf that was cut by choke-borer from the sample leaves. The leaves were measured for their fresh weight (FW), soaked in the distilled water for 24 hours, and then they were weighted to find water-saturated weight (WSW). Later on, the leaves were dried in a 80 ^oC oven for 3 days to find dry weight (DW). RWC was calculated as follows[9]:

$$RWC = ((WSW - FW)/(WSW - DW)) \times 100\% .$$

Photosynthesis was measured using Chlorophyll Fluorescence Analyser (Qubit systems) to analyse the maximum efficiency of photosynthesis *Fv/Fm*[10]. Leaf anatomy was analysed using light microscope with paraffin method[11] as well as using Transmission Electron Microscope (TEM)[12]. Paraffin method was carried out to determine leaf thickness in major vein, intervein, and leaf blade, and the area of bulliform cells; and to determine xylem diameter and the number of bulliform cells. The measurement using TEM was carried out to analyse the number of chloroplast and starch in the mesophyll and bundle sheath cells.

The experiment was arranged using completely randomized design with two factors and 3 replications. The first factor was environmental factor that contained two levels: well-watered (control) and drought stress, while the second factor was plant species which comprised two levels: *O. sativa* (rice) and *E. crusgalli* (Echinochloa). The collected data were then analyzed by t-test analysis using SPSS 15.

3. Results

3.1. Media water status

Drought treatment for 6 days caused reduction of media water content of all plants from nearly 30% before drought to an average of 13.40% for rice and 15.55% for Echinochloa. During this period, droughted plants exhibited stress and loss their turgor specified by wilting in all part of the leaves, while control plants were entirely turgid. Three days after re-watering, media water content increased back to the level similar to that of control plants (Figure 1). Water content recovery in the droughted treatments caused the plants refreshed again and recovered from wilted.

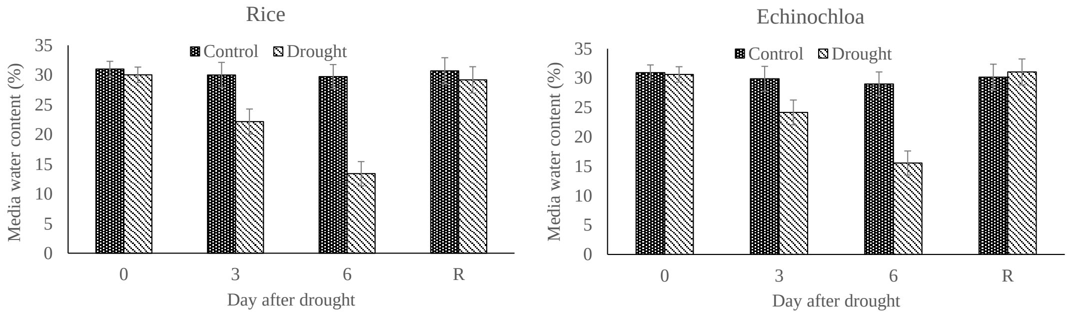


Figure 1. Media water content of rice and Echinochloa during 3 and 6 days of drought stress and 3 days after re-watering (recovery, R) between well-watered (control) and drought stress. (Bar indicative of standard error calculated from 5% of t-test).

3.2. Plant Growth

To compare plant growth of both species, several parameters including plant height, leaf number and tiller number were measured. Drought stress during 6 days caused the decrease of all growth component in both species including plant height, the number of leaves and tillers. However, based on statistical analysis, only plant height and the number of leaves of rice but not of Echinichloa that significantly decreased in response to drought treatment, while the others were not significantly different (Table 1).

Table 1. Plant height, leaf number and tiller number of rice and Echinochloa grown in well-water (control) and drought stress during 6 days.

Plants	Plant height (cm)		Leaf number		Tiller number	
	Control	Drought	Control	Drought	Control	Drought
Rice	81.30 a	75.00 b	12.40 a	10.00 b	6.10 a	4.40 a
Echinochloa	113.70 a	111.00 a	11.00 a	10.30 a	3.60 a	2.60 a

Note: The numbers followed by similar letter is not significantly different based on 5% of t-test analysis.

3.3. Relative water content

3.4. Drought stress exposed during 6 days caused the decrease of relative water content (RWC) of both species, even though each plant had different response. Leaf RWC of rice was declined significantly since 3 days after drought, and the decrease continued until 6 days of drought when it reached 54.73%, while it was still more than 92% for well-watered (control) plants (Figure 2). On the other hand, in Echinochloa, 3 days of drought slightly decreased RWC, even though it continued to decrease until 65.55% after 6 days of drought. During this time, both species were severely wilted suggesting that the plants were under severe stress. Three days after re-watering, both plants were able to recover from stress specified by the increase of RWC back to the level of almost similar to that of control plants (Figure 2).*Photosynthetic parameter*

Drought stress for 6 days which caused all plant to wilt surely affected plant photosynthesis. To analyze the impact of the stress to the photosynthetic apparatus, we observed the maximum efficiency of photosynthesis (Fv/Fm) using fluorescence analyzer. In general, drought stress caused the decrease of Fv/Fm of both species. After 6 days drought exposure the value of Fv/Fm declined significantly in both species. The decrease of Fv/Fm was high in droughted rice which reached 48.04% as compared to the control well-watered plants, even though it was almost unchanged when the drought was exposed for 3 days (Figure 3). In Echinochloa, 6 days drought caused the reduction of Fv/Fm by only 34.5%. Three days after re-watering, only Echinochloa which showed recovery indicated by the increase of Fv/Fm, while Fv/Fm of rice remained unchanged (Figure 3), suggesting that 6 days drought stress caused an impairment to the photosynthetic apparatus in rice.

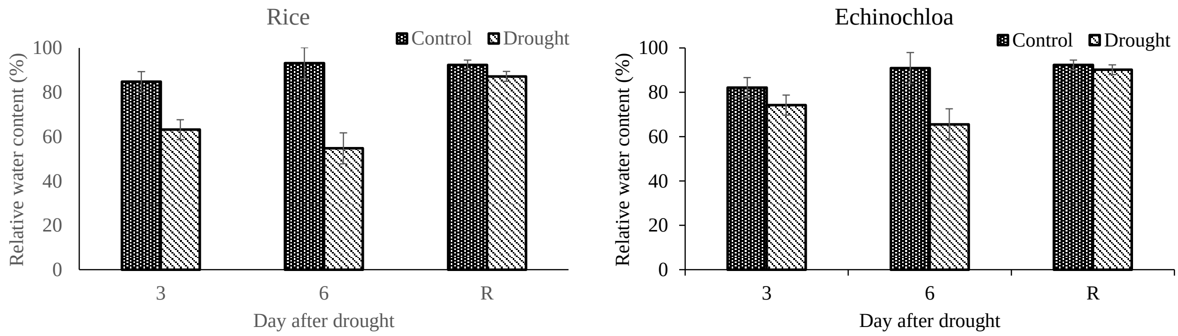


Figure 2. Relative water content (RWC) of rice and Echinochloa during 3 and 6 days of drought stress and 3 days after re-watering (recovery, R) between well-watered (control) and drought stress. (Bar indicative of standard error calculated from 5% of t-test).

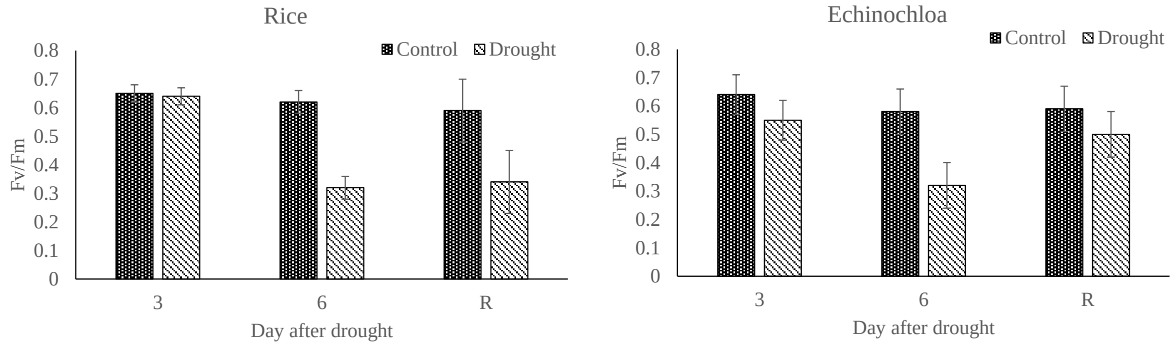


Figure 3. Maximum efficiency of photosynthesis (Fv/Fm) of rice and Echinochloa during 3 and 6 days of drought stress and 3 days after re-watering (recovery, R) between well-watered (control) and drought stress. (Bar indicative of standard error calculated from 5% of t-test).

3.5. Leaf Anatomy measurement

Leaf anatomy investigation was carried out by measuring leaf thickness, xylem diameter, and the bulliform cell number and height. Leaf thickness was measured in three area: major vein, bulliform cell and inter-vein. In general, drought treatment exposed during 6 days did not significantly influence leaf thickness of both species either at the area of major vein, bulliform cell as well as inter-vein (Table 2).

Table 2. Leaf thickness of rice and Echinochola in the are of major vein, bulliform cell and intervein in response to well-water (control) and drought treatments (stress).

Plants	Major vein (μm)		Change	Bulliform (μm)		Change	Intervein (μm)		Change
	Control	Stress	(%)	Control	Stress	(%)	Control	Stress	(%)
Rice	119.86	120.00	100.11	58.00	62.00	106.89	68/67	70.83	103.40
Echinoc	190.00	168.33	88.40	128.17	119.00	92.85	127.33	112.00	87.97

Note: The numbers followed by similar letter is not significantly different based on 5% of t-test analysis.

The analysis of xylem diameter of major vein and intervein was also not significantly affected by drought treatment for 6 days. Moreover, even though not significantly different, the decrease of xylem diameter due to 6 days drought stress tended to be higher in Echinochloa than in rice, and intervein xylem diameter tended to be affected more than major vein in both species (Table 3). The number and height of bulliform cell also remained unchanged due to drought stress. Despite not significantly different, however, the drought tended to slightly increase the number of bulliform cell in rice (table 4).

Table 3. The xylem diameter of rice and Echinochola in major vein and intervein in response to well-water (Control) and drought treatments (stress).

Plants	Major vein (μm)		Change (%)	Intervein (μm)		Change (%)
	Control	Stress		Control	Stress	
Rice	28.68 a	27.85 a	97.11	6.83 a	6.25 a	91.51
Echinochloa	32.64 a	29.44 a	90.20	8.42 a	6.50 a	77.20

Note: The numbers followed by similar letter is not significantly different based on 5% of t-test analysis.

Table 4. The number and height of bulliform cell of rice and Echinochola in response to well-water (Control) and drought treatments (stress).

Plants	Bulliform number		Change (%)	Bulliform height (μm)		Change (%)
	Control	Stress		Control	Stress	
Rice	10.34 a	12.95 a	125.24	21.05 a	20.97 a	99.70
Echinochloa	9.77 a	9.56 a	97.86	35.20 a	35.65 a	101.27

Note: The numbers followed by similar letter is not significantly different based on 5% of t-test analysis.

TEM investigation was also carried out to analyze chloroplast and starch granules in the mesophyll and bundle sheath cell of rice and Echinochloa. The data showed that both mesophyll and bundle sheath cells contained a lot of chloroplast in Echinochloa, while in rice chloroplast it was found only in mesophyll cell. Drought stress for 6 days caused the increase of chloroplast in bundle sheath cell of Echinochloa. The drought also tended to increase the number of chloroplast in mesophyll cell of both species, even thought it was not significantly different (Table 5).

The starch granules inside the chloroplast (chloroplastic starch) was also altered in response to 6 days drought treatment. Drought caused the increase of chloroplastic starch in rice up to 9 fold as compared to that of control plants. In Echinochloa the significant increase of chloroplastic starch also occurred not only in the mesophyll but also in the bundle sheath cell in response to drought treatment. The drought improved chloroplastic starch almost 5 times and 3 times in the mesophyll and in the bundle sheath cell of Echinochloa, respectively (Table 6 and Figures 4 and 5).

Table 5. The number of chloroplast in the mesophyll and bundle sheath cell of rice and Echinochola in response to well-water (Control) and drought treatments (stress).

Plants	Mesophyll cell		Change (%)	Bundle sheath cell		Change (%)
	Control	Stress		Control	Stress	
Rice	5.4 a	5.8 a	107.40	0	0	0
Echinochloa	5.2 a	5.8 a	111.53	5.4 a	7.8 b	144.44

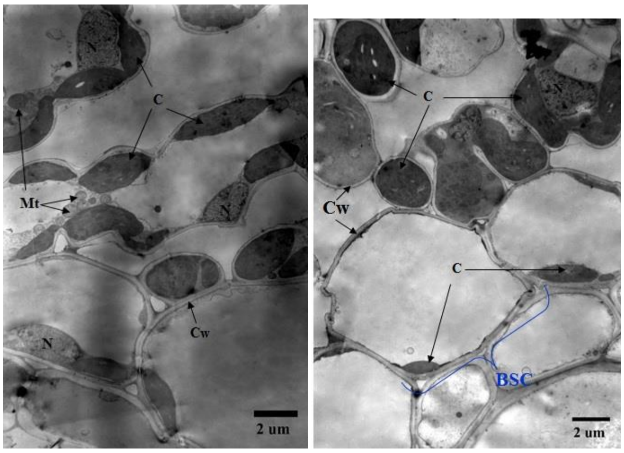
Note: The numbers followed by similar letter is not significantly different based on 5% of t-test analysis.

Table 6. The number of chloroplastic starch in the mesophyll and bundle sheath cell of rice and Echinochola in response to well-water (Control) and drought treatments (stress).

Plants	Mesophyll cell		Change (%)	Bundle sheath cell		Change (%)
	Control	Stress		Control	Stress	
Rice	0.6 a	5.4 b	900.00	0	0	0
Echinochloa	1.2 a	5.8 b	483.33	6.0 a	16.2 b	270.00

Note: The numbers followed by similar letter is not significantly different based on 5% of t-test analysis.

Rice



Echinochloa

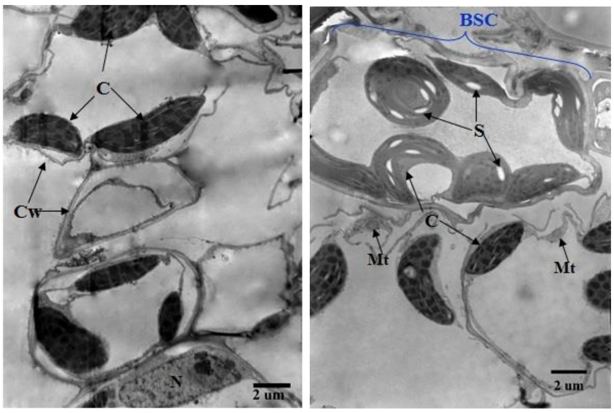


Figure 4. Electron micrograph of chloroplasts of rice and Echinochloa in the mesophyll cell (left) and bundle sheath cell (right) (3000x of magnification). Chloroplast (c), Nucleus (N), Bundle Sheath Cell (BSC), Mitochondria (Mt), Cell wall (Cw).

Rice

Echinochloa

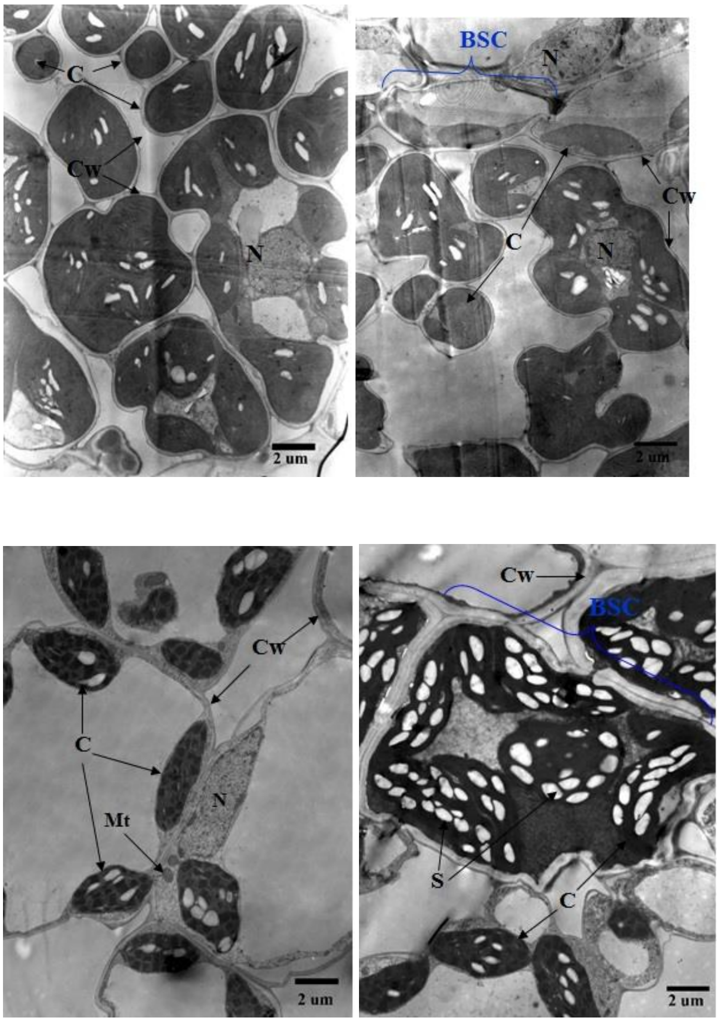


Figure 5. Electron micrograph of chloroplastic starch of rice and Echinochloa in the mesophyll cell (left) and bundle sheath cell (right) during well-watered and drought stress (3000x of magnification). Chloroplast (c), Nucleus (N), Bundle Sheath Cell (BSC), Mitochondria (Mt), Cell wall (Cw).

Discussion

3.6. Growth, physiological and anatomical responses of C3 and C4 plant to drought stress

Drought stress becomes major constraint of plant production, because drought influences many aspects inside the plant starting from cellular until organism level including physiology, metabolism, anatomy and even morphology. In physiological aspect, drought will decrease plant water potential which has negative impact on cell expansion and consequently plant growth[13]. The decrease of plant water potential due to drought also triggers the reduction of stomatal conductance which results in the decrease of photosynthetic CO_2 assimilation[4]. From these two aspects, it become understandable that drought or water stress reduces plant growth and production drastically, especially in rice[14].

In this experiment, drought stress provided by withholding water for 6 days significantly reduced media water content of rice and Echinochloa plants in average up to 50% of control (Figure 1). MWC of rice was slightly lower than that of Echinochloa in response to the last period of drought. This stress also decreased RWC of both rice and Echinochloa, but the decrease was bigger in rice than in

Echinochloa (Figure 2). Consequently, the drought caused the decline of maximum efficiency of photosynthesis (*Fv/Fm*) of rice bigger than Echinochloa (Figure 3). Plant growth data also confirmed that drought significantly inhibited growth of rice indicated by plant height and leaf number, but not Echinochloa (Table1).

To eliminate the effect of different water status that was happen during drought treatment, we analyze correlation between MWC and RWC of both species, so that we can evaluate the physical and physiological response of both species to the drought (Figure 6). From the graph shown in Figure 6, we conclude that the decrease of RWC in response to MWC declining was higher in rice than in Echinochloa. It means that drought caused rice plant underwent stress faster than Echinochloa, because RWC is an important physiological parameter of stress due to water deficit. The capacity of plant to maintain RWC during drought stress is an important tolerant-characteristic[15]. Some tolerant plants sometimes have capacity to avoid the stress by maintaining RWC with the mechanism known as osmotic adjustment[16].

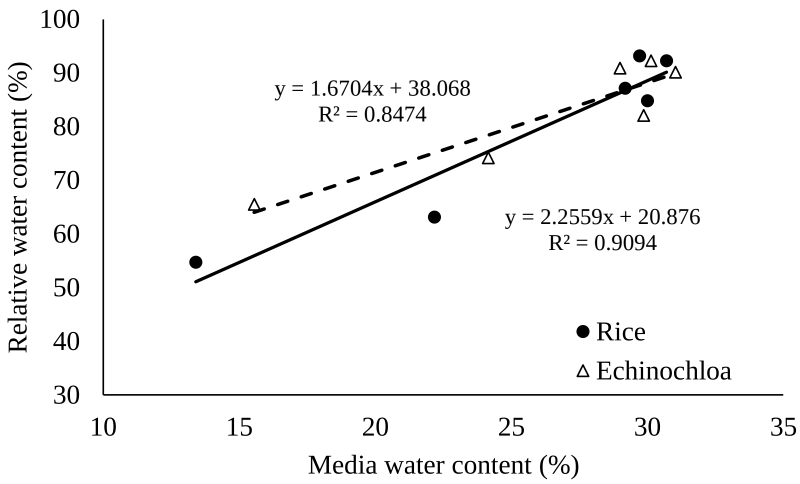


Figure 6. Correlation of media water content (MWC) and relative water content (RWC) of rice and Echinochloa during drought treatment. RWC of rice was decrease higher than that of Echinochloa in response to decrease of MWC.

The question was arise whether this difference has correlation with anatomical characteristics of both species. Xylem diameter, for example, is among the parameter that is suggested to have a role in drought tolerance[17]. This may associated to the development of cavitation and embolisms due to drought stress which cause the plant undergo stress[18]. An analysis on some tree species in Africa suggested that the trees with smaller vascular tissues were less vulnerable to cavitation and embolism and thus more tolerant of drought indicated by lowering dieback[19]. Anatomical analysis indicated that during drought treatment xylem diameter of Echinochloa either in major- as well as intervein was more elastic and decreased greater than that of rice (Table 3). This data also associated to the decrease of leaf thickness in the area of major vein as well as bulliform cell and intervein of Echinochloa that was greater than that of rice (Table 2). These were probably the reasons why Echinochloa underwent less stress than rice in response to 6 days drought.

3.7. Chloroplast and starch accumulation in response to drought stress

Plants usually response water stress by complex mechanisms including alteration of morphology and anatomy as well as physiology and metabolism. Chloroplast and starch accumulation inside the chloroplast is among several mechanisms observed in this experiment. The data clearly verified the existing chloroplast in the bundle sheath cell of Echinochloa, while it was absent in that of rice, suggesting that Echinochloa is a C4 species, and rice is C3 species. This is also in agreement with the classification of C4 species made by Sage and Manson[20] that Echinocloa is a C4 species. Interestingly, the number of chloroplast increased significantly in the bundle sheath cell of Echinochloa but not in mesophyll cell, during the drought stress (Table 5). This may associated with the increase of metabolic (carbohydrate) that was required to produce various substances to overcome stress. Several compounds such as trehalose, glycine betaine and proline are among the osmolyte substances abundantly accumulated during drought stress in many species that have function as osmoprotectant[16].

Starch produced during photosynthesis is a dynamic substance which is temporally accumulated in the chloroplast or exported from mesophyll cell to other cell of the plant through vascular tissues[21]. We compared chloroplastic starch accumulation in these two species to understand the different carbohydrate metabolism between C3 and C4 species in response to drought stress. Interestingly, in Echinochloa, starch was existed in mesophyll (even though only minor) as well as in bundle sheath cell (Table 6 and Figure 5). The existence of starch granules in mesophyll as well as bundle sheath cell of Echinochloa suggesting that C3 pathway (Calvin cycle) may be happened together with C4 pathway in Echinochloa. The C3-like characteristic of C4 species have been discussed by many authors as a specific phenomenon in C4 species such as *Panicum*[22]. The substantial increase of chloroplastic starch due to drought also occurred in mesophyll cell as well as in bundle sheath cell of Echinochloa as it was happened in mesophyll cell of rice (Table 6). This also even becomes strong evidence that C3 cycle might be occurred in mesophyll cell of Echinochloa (C4 species) especially during drought stress.

Conclusion

Rice exhibited more stress than Echinocloa due to drought stress indicated by the reduction of leaf relative water content, maximum efficiency of photosynthesis and plant growth. Drought treatment tended to reduce leaf thickness in the area of bulliform cell, major- as well as intervein and xylem diameter more in Echinochloa than in rice, suggesting that the decrease of vein and xylem diameter is among the anatomical parameters that is important to overcome from drought stress in Echinochloa. The drought also significantly increased the number of chloroplast in bundle sheath cell of Echinochloa, but not in mesophyll cell. The number of starch granules also increased in response to drought in mesophyll cells of rice and Echinochloa and in bundle sheath cell of Echinochloa, an indication that C3 cycle might occurred in C4 species at least in Echinochloa, especially during drought stress.

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