Effect of various drought stresses and subsequent recovery on proline, total soluble sugar and starch metabolisms in Rice (*Oryza sativa* L.) varieties

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**ABSTRACT**

Rice (*Oryza sativa* L.) is one of the most important staple foods in the world, however most improved rice varieties are susceptible to drought stress. A two-year study was conducted to explore the effects of various drought stresses and subsequent recovery on the accumulation and degradation of proline, total soluble sugar and starch in different rice varieties at vegetative stage. The results showed that relative water content in the leaves and sheaths of rice varieties significantly decreased under drought stresses, but not at the same rate. Under control and drought conditions, the water content in sheaths was higher than that in leaves. Interestingly, under severe drought stress in 2015, the leaf water content was higher than the sheath water content. The water distribution between leaves and sheaths might be a response of plants to protect leaf system from devastation by drought. Proline was highly accumulated under drought stress but rapidly decreased after re-watering. The drought tolerant variety DA8 expressed higher ability in accumulation of proline than susceptible varieties. In general, total soluble sugar and starch contents in leaves and sheaths of varieties decreased under drought stress conditions. Total soluble sugar and starch content of DA8 were less affected than other varieties under drought conditions. Our study indicated that metabolisms of total soluble sugar and starch in rice were affected by both environmental conditions and characteristics of varieties. Proline accumulation ability of varieties can be used as a useful indicator for drought tolerant potential in rice breeding for water-limited environments.
1. Introduction

Rice (Oryza sativa L.) is the staple food for more than half of the world’s population, especially people in developing countries (Seck et al., 2012). Although drought is one of the most severe stresses impeding rice production, most improved rice varieties are susceptible to drought. Rice breeding for drought tolerance is considered as a promising approach to increase rice yield in waterprone environments. This approach requires an understanding about drought-tolerant mechanism in rice plant.

Plants have evolved a sophisticated and complex set of mechanisms to cope with environmental stresses. Overproduction of various compatible organic solutes is one of the most common stress responses of plants to environmental stresses (Serraj & Sinclair, 2002). Proline accumulation is a well-known metabolic response of plants to drought and other stresses (Szabados & Savouré, 2010). Proline permits osmotic adjustment, stabilizes the structure of proteins and cell membranes, acts as a protective agent for enzymes, and is a free radical scavenger and antioxidant (Ashraf & Foolad, 2007; Kishor & Sreenivasulu, 2014; Verbruggen & Hermans, 2008). Some studies have shown that proline accumulation is generally an indicator of leaf dehydration and is associated with stress susceptibility (Hanson et al., 1977). It was found that proline was highly accumulated in drought-susceptible than drought-resistant potato genotypes (Bansal & Nagarajan, 1986; Schaifeitner et al., 2007). In contrast, drought-resistant genotypes of cotton, tall fescue and wheat were characterized by higher proline accumulation than the susceptible ones (Man et al., 2011; Parida et al., 2008; Sultan et al., 2012). In rice, the concentration of proline was remarkably increased during drought stress (Mostajar en & Rahimi-Eichi, 2009). Abdula et al. (2016) found that the increasing of proline biosynthesis enhanced abiotic stress tolerance in rice varieties. However, Bing-Sheng et al. (2014) suggested that proline accumulation is not correlated with salt, alkaline and osmotic stresses in rice. Bandurska et al. (2017) found that the difference in proline accumulation was not affected drought tolerance in barley genotypes. Therefore, despite extensive researches on proline accumulation under water deficit conditions, there are still controversial opinions about the actual correlation between proline accumulation and drought resistance in plants.

Soluble sugars (sucrose, glucose and fructose) play an important role in maintaining the overall structure and growth of plants (Rosa et al., 2009). Lemoine et al. (2013) suggested that soluble sugar regulation in plants was a very complex manner. Soluble sugar maintains the leaf water content and osmotic adjustment of plants facing the conditions of drought stress (Koster & Leopold, 1988; Xu et al., 2007). Xu et al. (2015) found that drought stress condition significantly increased soluble sugar concentration in roots and leaves of susceptible rice variety but not in resistant one. In contrast, stem soluble sugar concentration in both susceptible and tolerant genotypes was significantly reduced under drought stress (Xu et al., 2015). In relationship with proline metabolism, soluble sugar accumulation enhanced proline content under salt stress (Hellmann et al., 2000). It is believed that study of sugar under various abiotic stresses is an emerging field of research, and it could play pivotal role in tolerance against abiotic stresses by modulating several physiological processes (Rathinasabapathy, 2000).

Starch is also emerging as a key molecule in mediating plant responses to abiotic stresses, such as water deficit, high salinity or extreme temperatures. Under these challenging environmental conditions, plants generally remobilize starch to provide energy and carbon at times when photosynthesis may be potentially limited. The released sugars and other derived metabolites support plant growth under stress, and function as osmoprotectants and compatible solutes to mitigate the negative effect of the stress (Krasensky & Jonak, 2012). Leaf starch content was reported to decrease in response to abiotic stress, independently of the analyzed species (Thalmann & Santelia, 2017). Degradation of starch in response to stress often has been correlated with improved tolerance. Cruz and Pastenes (2012) found that a drought-resistant variety of broad bean (Phaseolus vulgaris) degraded more starch than a drought-sensitive variety. Matthias Thalmann et al., (2016) proposed that the regulation of leaf starch degradation was important for osmotic stress tolerance in plants. In contrast, several studies also reported an increase in starch accumulation under stress (Kaplan & Guy, 2004; Siaut et al., 2011; Skirycz et al., 2010; Wang et al., 2013).

Although effects of water stress on plants alone have been well documented in many researches, the combined responses of rice to drought and subsequent recovery conditions are relatively scant. In addition, the intensity of drought stress is often different from year to year and within fields because of variation in soil composition which determines the capability of the soil to retain water. While the drought stress tends to develop slowly as the soil gradually dries, many researches applied ‘shock’ treatments when immediately transfer plants from well-watered condition to severe drought stress condition. In this study, drought stresses were slowly developed and followed the soil
drying through the experimental time. When drought stress was reached to moderate and severe levels, water is applied to verify the recovery ability of varieties, respectively. This two-year study aimed to explore the response of different varieties under various drought stresses and subsequent recovery conditions in terms of proline, total soluble sugar and starch metabolism. The variation in accumulation and degradation activities of proline, total soluble sugar and starch content were intensively analysed. The important roles of proline, total soluble sugar and starch in drought tolerance in rice are also discussed in this study.

2. Materials and methods

2.1. Experimental design

In this study, two experiments were conducted in a greenhouse of Faculty of Agriculture, Kyushu University, Japan (33°37‘N, 130°25‘E, 3 m above the sea level) in summer 2015 and 2017, respectively.

In 2015 experiment, a pot experiment was conducted using six rice varieties included DA8, Malagkit Pirurutong, Thierno Bande, Pate Blanc MN1, Kinandang Patong and Moroberekan. DA8 and Thierno Bande expressed as drought tolerant varieties while Malagkit Pirurutong and Pate Blanc MN1 were drought sensitive varieties in our previous study (Dien et al., 2013). Kinandang Patong and Moroberekan were defined as drought tolerant varieties in previous studies (Dixit et al., 2014; Uga et al., 2013) and used as the checked varieties in this experiment. Plants were cultivated in Wagner pots (1/5000a) contained 3.0 kg of air-dried Futsukaichi soil (sandy loam soil, water-holding capacity was 47.8%). To each pot, fertilizers were applied at rate of 0.2 g N + 0.2 g P₂O₅ + 0.2 g K₂O at 2 days before sowing day. In this experiment, (NH₄)₂SO₄, K₂SO₄ and KH₂PO₄ were used as the forms of fertilizers. The above chemicals were dissolved in distilled water and applied by mixing with the soil of each pot. Soil had been sieved through 2-cm mesh screen and pre-mixed with fertilizer at 2 days before sowing time.

Drought treatments were started at 4 weeks after sowing by withdrawing water from drought treatment pots while retaining a water level of 2 cm above the soil surface in control pots. The drought treatments were 10 days and 15 days, respectively. After the drought treatment, plants were re-watered to permit recovery.

There were five sampling times included 1 day before drought treatment (BDT), 10 days after drought treatment (moderate drought), 15 days after drought treatment (severe drought), 7 days after re-watering from moderate drought and 7 days after re-watering from severe drought. For each sampling time, samples of leaves and sheaths were separately collected from 10:00 to 13:00, quickly placed in pre-weighed zip-sealed bags, and measured immediately to determine fresh weights. Leaves used for proline analysis were then freeze-dried for 48 h by freeze dryer FDU-506 (EYELA, Tokyo Rikakikai Co., LTD., Tokyo, Japan). Other samples were transferred to paper bags and dried at 70°C for 48 h by an electric drying oven (DRM 620TB, Advantec, Tokyo, Japan).

Based on responses of varieties in 2015 experiment, three rice varieties (DA8, Malagkit Pirurutong and Kinandang Patong) were selected for a pot experiment in 2017. In which, DA8 was a drought tolerant variety, Malagkit Pirurutong was a drought sensitive variety and Kinandang Patong expressed as the fastest recovery variety among varieties in 2015 experiment (Dien, Mochizuki, et al., 2017). In order to remain the same drought stress level among varieties, plants of three varieties were simultaneously cultivated in a same pot. The 1/2000a Wagner pots, which contained 12 kg air-dried soil was used. Fertilizer level applied for each pot was 1.0 g N + 1.0 g P₂O₅ + 1.0 g K₂O. Forms of fertilizers and application method in 2017 were same as in 2015 experiment. Drought treatment was started at 4 weeks after sowing with the same method in 2015. Moderate drought and severe drought in 2017 were verified at 6 and 9 days after drought treatment, respectively. There were seven sampling times in 2017 experiment, included five times as the same in 2015, and two more sampling times at 1 day re-watering after moderate drought and 1 day re-watering after severe drought. For each sampling time, leaves and sheaths were separately collected from 10:00 to 13:00, quickly placed in pre-weighed zip-sealed bags, and measured immediately to determine fresh weights. Collected samples were freeze-dried for 48 h by freeze dryer FDU-506 (EYELA, Tokyo Rikakikai Co., LTD., Tokyo, Japan) before measured for dry weight and used for proline, soluble sugar and starch analyses.

Soil water content in pots was measured by EC 5 soil moisture sensors (Decagon, Pullman, USA). The soil moisture sensors were set with the tips of sensors at the middle point between plant and pot border, 10 cm below the soil surface. In principle, sensors measured the dielectric constant of bulk soil and then converted these data to the values of volumetric water content. The recording interval time was 30 min, and then raw recorded data were averaged for each day. Air humidity and air temperature inside the greenhouse were measured using a TR 72wf Thermo Recorder (T&D Corporation, Nagano, Japan). Sensors of the TR 72wf Thermo Recorder were set at the same height of
plant canopy, nearby the experimental pots. Soil temperature in control and drought pots were recorded by a TR 71U Thermo Recorder (T&D Corporation, Nagano, Japan). Sensors of the TR 71U Thermo Recorder were set at the middle point between the plant and pot border, 10 cm below the soil surface. Recording interval time of parameters was 30 min, and then raw recorded data were averaged for each day. Average values of air humidity, air temperature and soil temperature during study periods are shown in Figure 1.

2.2. Determination of relative water content

The relative water content (%) in the leaves and sheaths of each variety was calculated based on the equation:

\[
\text{Relative water content} (\%) = \frac{(\text{Fresh weight} - \text{Dry weight})/\text{Fresh weight}}{\times 100}
\]

2.3. Determination of proline content

Samples were ground by a grinder (Heiko Sample Mill, Heiko Seisakusho, LTD., Tokyo, Japan) before being used for proline content analyses. The extraction step was conducted with the following procedure: 10 mL of hot 80% ethanol was added in 100-mL conical flask containing 0.1 g of dried leaf powder sample and was heated on the hotplate controlled 80°C. The extracted materials were poured into a 50-mL volumetric flask through a funnel with 1 layer of filter paper (5B, ADVANTEC Corp., Tokyo, Japan). Conical flask and funnel were rinsed by 10 mL of hot 80% ethanol for 4 times. Fill up 50 mL of volumetric flask by 80% ethanol. For proline analysis, 10 mL of extract was taken into a 50-mL test tube. The test tube was added 2 mL of acid ninhydrin (1.25 g ninhydrin in 30 mL glacial acetic acid and 20 mL of 6 M phosphoric acid) and 5 mL of glacial acetic acid and placed in boiling water bath for 45 min. After that, the test tubes were cooled in an ice bath for 5 min. The content was vigorously mixed with 10 mL of toluene. Mixture was warmed up to room temperature, and the upper layer was measured at 520 nm using toluene as blank. The proline concentration (µmol g\(^{-1}\) D.W) is determined using a proline standard curve (Bates et al., 1973). In 2015, proline content was analysed for second-top-fully-expanded leaves only. In 2017, proline in fully-expanded leaves and sheaths of varieties were analysed.

![Figure 1](image_url). Environmental temperature and humidity during study period in 2015 and 2017 years.
2.4. Determination of starch and total soluble sugar content

Quantification of soluble sugars and starch content in leaves and sheaths in both years were carried out follow the method of Yoshida et al. (1973). Samples were ground by a grinder (Cyclotec™ 1093, FOSS, Denmark) before used for extraction. For the extraction step, 50 mg of dried-ground sample was added into a 15-mL centrifuge tube and added 5 mL of 80% ethanol. Placed a glass ball on top of the tube and keep in a water bath at 80–85°C for 30 min. Centrifuged at 3,000 rpm for 10 min and decanted into a 50-mL volumetric flask, kept the residue in centrifuge tube. Repeated this extraction three more times. The supernatant in 50-mL volumetric flask was then filled up by 80% ethanol. This extract was used for total soluble sugar content. The residue in the test tube was dried at 80°C for 1 h. 1.0 mL of distilled water was added into the tube; waiting for completely absorption. The tube was put in a boiling water bath for 15 min. 1 mL of 9.2 N HClO₄ was added into the tube; stirred occasionally for 15 min by vortex. The suspension was then made up to about 5 mL by distilled water and centrifuged at 3,000 rpm for 10 min. The supernatant was decanted to a 50-mL volumetric flask. 1 mL of 4.6 N HClO₄ was added to residue in test tubes. This suspension was stirred for 15 min by vortex. Centrifuged and decanted the supernatants to the 50-mL volumetric flask, and repeated the extraction with 4.6 N HClO₄ one more time. Then combined the supernatants and filled up the volumetric flask by distilled water.

For total soluble sugar analysis, 0.5 mL of soluble sugar extract and 4.5 mL of 80% ethanol were added into a test tube. Put the sample tubes into an ice bath and slowly added 10 mL of anthrone reagent to the tubes. Put the tubes in a boiling water bath for exactly 7.5 min and then immediately cooled in an ice bath. After cooling, the absorbance at 630 nm in 1 h was measured.

For starch analysis, 0.5 mL of starch extract and 4.5 mL of distilled water were added into a test tube. Put the test tube into an ice bath and then 10 mL of anthrone reagent was slowly added into the test tube. Put the tubes in a boiling water bath for exactly 7.5 min before immediately cooled in an ice bath. After cooling, the absorbance at 630 nm in 1 h using a spectrophotometer UV-120–02 (Shimadzu, Nagoya, Japan) was measured.

2.5. Statistical analysis

The experiment was conducted in a randomized complete block design with three replications. Analysis of variance was used to test for differences, and Turkey’s HSD test was used to calculate the significant difference at the 5% probability level using STAR 2.0.1 software.

3. Results

3.1. Climate data

The climate data in the experimental greenhouse during study periods were observed for both years 2015 and 2017. In comparison between 2 years, air temperature, soil temperatures under control and drought conditions in 2017 (with average values were 31.4°C, 33.3°C and 33.5°C, respectively) were higher than those in 2015 (with average values were 25.8°C, 26.4°C and 26.8°C, respectively). In contrast, air humidity in 2015 (69.6%) was higher than that in 2017 (63.8%) (Figure 1). Because of higher temperature and lower humidity, drought stress developed in 2017 was faster than in 2015. In 2015, moderate drought was verified at 10 days after drought treatment and severe drought was 15 days after drought treatment. The times for moderate drought and severe drought in 2017 were 6 and 9 days after drought treatment, respectively.

3.2. Soil water content

Volumetric water content (v/v) during drought treatment in 2015 and 2017 are shown in Figure 2. In 2015, there were six different varieties and each variety was separately cultivated in an experimental pot. There were three rice varieties in 2017 including DA8 (drought tolerant), Malagkit Pirurutong (drought sensitive) and Kinandang Patong (fast recovery). These three varieties were simultaneously cultivated in a same pot to remain the same soil moisture content between varieties. In both years, soil water content was remarkably decreased through the time of drought treatment. In comparison between experimental varieties of 2015, Moroberekan remained the highest soil water content while Thierno Bande expressed the lowest soil water content during drought treatment. At 10 and 15 days after drought treatment, average volumetric water content of varieties were 0.087 and 0.073 cm⁻³/cm³, respectively. There was no significant different in soil water content between DA8, Malagkit Pirurutong and Kinandang Patong at 10 and 15 days after drought treatment.

Similar to 2015, soil water content in 2017 was sharply decreased after drought treatment. Soil water content at 6 and 9 days after drought treatment were 0.116 and 0.102 cm⁻³/cm³. In comparison with soil water content at 6 and 9 days after drought treatment in 2015, there was no significant difference in soil water content between 2 years.

3.3. Relative water content in leaves and sheaths of varieties

The relative water content (%) in the leaves and sheaths of the experimental varieties in both 2015
and 2017 decreased significantly under drought conditions compared with control condition (Figure 3). In 2015, the average leaf water content of all varieties declined dramatically from 78.59% at before drought treatment to 41.19% under moderate drought stress and 20.16% under severe drought stress. Before drought treatment, the leaf water contents of DA8 and Thierno Bande, the drought-tolerant varieties, were lower than those of the other varieties. Leaf water content did not differ significantly among varieties under moderate drought. However, under severe drought, Pate Blance MN1, a drought-sensitive variety, expressed the highest leaf water content among the varieties. In 2017, there was no significant different in leaf water content between varieties under control condition. However, under moderate drought, DA8 showed the highest leaf water content (60.62%), followed by Kinandang Patong (47.12%) and Malagkit Pirurutong (23.42%). The order remained until severe drought, with the leaf water content of varieties were 19.92% for DA8, 9.30% for Kinandang Patong and 7.04% for Malagkit Pirurutong, respectively.

The average sheath water content of varieties in 2015 sharply decreased from 82.69% at before drought treatment to 57.74% at moderate drought, and fell significantly to the lowest value (9.84%) at severe drought. DA8 and Thierno Bande normally expressed lower sheath water contents than other varieties in 2015. Before drought treatment and moderate drought in 2017, there was no significant difference in sheath water content between varieties. The average sheath water content of three varieties was 88.80% at before drought treatment and 68.00% under moderate drought, respectively. Under severe drought, DA8 remained highest sheath water content (68.08%) compared to Kinandang Patong (52.86%) and Malagkit Pirurutong (24.49%).

In comparison between leaves and sheaths under the same condition, leaf water content of varieties generally remained lower than sheath water content in both 2015 and 2017, except for severe drought condition in 2015 where leaf water content significantly higher than sheath water content.

### 3.4. Proline content

Under control condition, there was no significant difference in leaf proline and sheath proline content between experimental varieties in both years of experiments. However, there was a large variation in proline accumulation between varieties under moderate and severe droughts (Figure 4).

Under moderate and severe droughts of 2015 experiment, leaf proline content was highest in DA8 (24.13 and 27.67 μmol g\(^{-1}\) DW, respectively), followed by Therno Bande (16.13 and 16.86 μmol g\(^{-1}\) DW, respectively); these proline contents were significantly higher than those in other varieties (Figure 4(a)). The leaf proline content of DA8 increased significantly from 24.13 μmol g\(^{-1}\) DW under moderate drought to 27.67 μmol g\(^{-1}\) DW under severe drought. Similarly, an increase in the leaf proline content of Moroberekan was observed, from 6.53 μmol g\(^{-1}\) DW under moderate drought to 9.17 μmol g\(^{-1}\) DW under severe drought.

Similar to 2015, proline content in leaves of varieties in 2017 were significantly increased under drought stress conditions compared to control condition (Figure 4(b)). In both moderate drought and severe drought, leaf proline content of DA8 (24.60 and 30.11 μmol g\(^{-1}\) DW, respectively) was significantly higher than those in Malagkit Pirurutong (8.58 and 8.82 μmol g\(^{-1}\) DW, respectively) and Kinandang Patong (9.26 and 11.25 μmol g\(^{-1}\) DW, respectively). When drought stress increased from moderate to severe drought, DA8 was the only variety which leaf proline content significantly
increased from 24.60 to 30.11 µmol g\(^{-1}\) DW. Sheath proline content of varieties in 2017 significantly increased under moderate and severe droughts compared to control condition (Figure 4(c)). In comparison with Malagkit Pirurutong and Kinandang Patong, DA8 showed the highest accumulation of sheath proline under moderate drought (11.20 µmol g\(^{-1}\) DW) and severe drought (12.14 µmol g\(^{-1}\) DW), respectively. Under the same drought stress condition, accumulation of proline was higher in leaves than in sheaths. One day after re-watering, proline content in leaves and sheaths of varieties was rapidly decreased and significantly lower than those under respective drought stress. However, proline content at 1 day after re-watering still remained higher than those under control condition, except for sheath proline content in Kinandang Patong at 1 day after re-watering. One day re-watering after moderate drought in 2017, sheath proline content of Kinandang Patong decreased to the same level of control condition (Figure 4(b,c)). Proline content of almost in all varieties decreased to the control level at 7 days after re-watering (Figure 4(a–c)).

The relationship between water content and proline accumulation of rice varieties in both 2015 and 2017 is shown in Figure 5. It was obvious that proline accumulation of rice varieties negatively correlated with water content in varieties. Generally, more severe drought stress resulted in lower water content, and consequently higher proline accumulation. However, the regression functions were different between varieties. In 2015, at the same water content, DA8 and Thierno Bande expressed higher proline content than other varieties. DA8 remained higher proline accumulation ability than Malagkit Pirurutong and Kinandang Patong in 2017 experiment (Figure 5).

**Figure 3.** Relative water content in leaves and sheaths of varieties under different water conditions in 2015 and 2017 years. Means with same letters are not significantly different between varieties in the same condition (n = 3; P < 0.05). ns: not significant, asterisk (*) represents significant differences of water content between leaves and stems for each variety at 5% level, respectively. Values are mean ± SE (n = 3).
3.5. Soluble sugar content

Soluble sugar content in leaves and sheaths of varieties were analysed in both 2015 and 2017 (Figure 6). Under control condition, soluble sugar content fluctuated through the experimental time. Before drought treatment in 2015, leaf soluble sugar content was high in DA8, Blanc MN 1 and Moroberekan. DA8 also remained the highest leaf soluble sugar before drought treatment in 2017. Moderate drought significantly decreased leaf soluble sugar of varieties in both years, except for DA8 in 2015. Similarly, severe drought stress negatively affected leaf soluble sugar content of varieties in both years, except for DA8 and Blanc MN1 in 2015. In 2015, leaf soluble sugar content in Malagkit Pirurutong, Blanc MN1 and
Moroberekan at 7 days re-watering after moderate drought retained significantly lower than those under control condition (Figure 6(a)). There was no significant difference in leaf soluble sugar content between control and treatment pots at 1 day re-watering after moderate drought and days re-watering after moderate drought in 2017 (Figure 6(b)). In 2017, leaf soluble sugar content of varieties was not significantly different under control condition, except for the time of before drought treatment and 7 days re-watering after severe drought. At 1 day re-watering after severe drought, leaf soluble sugar of varieties still remained significantly lower than those under control condition. In contrast, DA8 accumulated higher amount of soluble sugar at 7 days re-watering after severe drought than control condition. At 7 days re-watering after severe drought, DA8 expressed higher soluble sugar content, Malagkit Pirurutong accumulated lower soluble sugar while there was no significant in case of Kinandang Patong, compared to control condition. This response was consistent in both 2015 and 2017.

Soluble sugar in sheaths of varieties under moderate drought was not significantly different compared to control condition, except for DA8 at 2015, which was significantly higher than that under control (Figure 6(c)). At 1 day re-watering after moderate drought in 2017, only Malagkit Pirurutong showed the lower sheath soluble sugar content compared to control condition. At 7 days re-watering after moderate drought, DA8 and Malagkit Pirurutong decreased sheath soluble sugar compared to control condition, but not for Kinandang Patong. In 2015, sheath soluble sugar of all varieties was lower at 7 days of re-watering after moderate drought than control condition. Severe drought significantly decreased the...
accumulation of soluble sugar in sheaths of varieties in both 2015 and 2017. At 1 day re-watering after severe drought, sheath soluble sugar of varieties still remained lower than those compared to control condition. Under control condition, sheath soluble sugar of varieties in 2017 was lower than those in 2015.

3.6. Starch content

Before drought treatment in both years, there was no significant difference in leaf starch and sheath starch content between varieties (Figure 7). In comparison to control condition, moderate drought increased leaf starch of DA8 and Malagkit Pirurutong in 2015, not
significantly changed sheath starch of Kinandang Patong in 2017, while decreased starch content in leaves and sheaths of other varieties in both years. At 1 day re-watering after moderate drought in 2017, leaf starch of all varieties remained lower than control condition. Similarly, sheath starch of Malagkit Pirurutong and Kinandang Patong was lower than those in control condition. However, DA8 expressed higher value of sheath starch compared to control condition. At 7 days re-watering after moderate drought,
except for DA8 in 2015 experiment, leaf starch content of varieties significantly lower than those in control condition. For the sheath starch content, re-watering of varieties accumulated lower starch content at 7 days after moderate drought compared to control condition in both years, except for Blanc MN1 in 2015 and Kinandang Patong in 2017.

Severe drought significantly decreased starch content in leaves and sheaths of all varieties, except for DA8 and Kinandang Patong in 2015. In 2017, starch content in leaves and sheaths of all varieties at 1 day re-watering after severe drought retained lower than those in control condition. At 7 days re-watering after severe drought in 2015, DA8 was the only variety, which accumulated higher leaf starch content than control condition. Sheath starch content in both years, leaf starch content in 2015 of varieties remained significantly lower compared to control condition.

4. Discussion

In this 2-year study, we analysed the effects of various drought stresses and subsequent recovery on the accumulation and degradation of proline, soluble sugar and starch content in different rice varieties at vegetative stage. In both 2015 and 2017, soil water content was dramatically decreased through the drought treatment time. In 2015, it was seen that the decreasing pattern of soil water content was differed slightly between varieties (Figure 2). This difference could be explained by the variation in phenotypes of varieties. In a previous study (Dien, Yamakawa, et al., 2017), DA8 and Thierno Bande exhibited bigger root systems compared to other varieties (with higher values of total root length, root surface area and root volume). This suggested that DA8 and Thierno Bande remained higher ability in water absorption than other varieties. Consequently, through the time of drought treatment, soil water content of DA8 and Thierno Bande was slightly lower than those in other varieties.

Rice is highly sensitive to water stress. Under drought stress conditions, roots absorb less water than under normal (well-watered) condition. Because of water deficit, water content in leaves and sheaths of varieties were significantly lower compared to control (well-watered) condition (Figure 3). In comparison between different parts of plant, relative water content in sheaths generally remained higher than in leaves under control and drought conditions in both years of study. However, under severe drought stress (at 15 days after drought treatment) in 2015, sheaths of varieties retained lower water contents than in leaves. These findings suggested that under normal and moderate drought stress conditions, water was primarily stored in sheath of rice plants. In contrast, water in plant was mainly transported to leaves than sheaths under severe drought stress condition. This indicated that the water distribution between parts of plants might be a response of rice varieties to protect their leaves from the devastation of severe drought stress. However, the mechanism, which controls water transportation between organs of rice plants under various drought stresses still was not clearly understood and must be explored in future studies.

In response to water deficit, osmotic adjustment is a biochemical mechanism that helps plants to acclimate to dry soil. One mechanism for osmotic adjustment is the accumulation of compatible solutes, such as the amino acid proline. Proline was first noted to accumulate in wilted plant tissue by Kemble and MacPherson (1954) in experiments on excised perennial rye grass. Many plants and microorganisms accumulate proline as a response to osmotic stress (Hare et al., 2002). Because the osmotic stress is faster and easier in leaves than sheaths or roots, previous studies mainly focus on proline metabolism in leaves only. The 2015 experiment in this study also focused on proline accumulation and degradation in leaves of six rice varieties. However, the experiment in 2017 concerned the proline metabolism in both leaves and sheaths of varieties. Proline content in leaves and sheaths of rice varieties was intensively analysed and shown in Figure 4. The results expressed that proline was highly accumulated in leaves and sheaths of rice plants under drought stress compared to control condition, and that more severe drought stress resulted to more proline accumulation. These results agreed to other previous studies, which mentioned that proline is highly accumulated under drought stress condition (Mostajeran & Rahimi-Eichi, 2009; Sultan et al., 2012; Szabados & Savouré, 2010). However, proline is highly accumulated in leaves than in sheaths, suggested that osmotic stress in leaves was more severe than in sheaths.

Under drought stress conditions, DA8 and Thierno Bande accumulated more proline than other varieties in 2015. In 2017 experiment, DA8 remained higher proline content than Malagkit Pirurutong and Kinandang Patong (Figures 4 and 5). Studies in transgenic plants have suggested that overproduction of proline enhances root biomass under drought in rice (Zhu et al., 1998). In our previous study, DA8 and Thierno Bande exhibited higher values for root dry weight and root morphological characteristics (total root length, root surface area, root volume) under drought conditions in comparison with other varieties (Dien, Yamakawa, et al., 2017). Larger root systems can help
plants absorb more water from the soil under drought stress conditions, and consequently more drought tolerant than other varieties. This result was consistent with our previous finding that DA8 and Thierno Bande were more tolerant to drought than other varieties. There was a strong-negative correlation between water content and proline accumulation in rice varieties in both year experiments (Figure 5). However, the regression functions were different among varieties. In both leaves and sheaths, with the same water content, DA8 and Thierno Bande expressed higher proline accumulation than those in other varieties in 2015 experiment. Similarly, DA8 showed higher proline accumulation ability than Malagkit Pirurutong and Kinandang Patong under the same water condition in 2017 experiment (Figure 5). The result suggested that proline accumulation in rice depended not only on the severity of drought stress but also on the characteristics of varieties. In addition, higher proline accumulation ability contributed to higher drought tolerance in rice. Therefore, high proline-accumulation ability might serve as an indicator for drought tolerance potential in rice.

After re-watering, the proline content of the rice varieties decreased rapidly, reaching values similar to those under the control condition (Figure 4). Several studies have indicated that proline accumulated during episodes of water deficit is lost rapidly when the water deficit is eliminated (Blum & Ebercon, 1976; Singh et al., 1973; Stewart, 1972). Once osmotic stress is withdrawn, proline is oxidised into \( \Delta^1 \)-pyrroline-5-carboxylate (PSC) by proline dehydrogenase, also known as proline oxidase, the first enzyme in the proline degradation pathway. Then, PSC is converted back into glutamate by the enzyme PSC dehydrogenase (Hare et al., 2002).

Although proline accumulation is a stress response, it depends on the availability and interactions of several effectors. Studies have indicated that soluble sugars are highly sensitive to environmental stresses, which act on the supply of carbohydrates from source organs to sink organs (Rosa et al., 2009). Several studies have shown that the soluble sugar content increases under drought stress. In contrast, a recent study of rice seedlings suggested that drought stress resulted in a remarkable reduction in soluble sugar accumulation in the whole plant (Xu et al., 2015). In this two-year study, soluble sugar content in leaves and sheaths of rice varieties was intensively analysed under different water regimes. Results from both years found that moderate drought negatively affected soluble sugar content in the leaves of rice varieties, except for DA8 in 2015. Severe drought stress significantly declined leaf soluble sugar of varieties, except for DA8 and Blanc MN1 in 2015 (Figure 6(a,b)). Similarly, soluble sugar content in sheaths of varieties was significantly decreased under severe drought stress compared with control condition in both 2015 and 2017 (Figure 6(c,d)). Soluble sugars, including monosaccharides and oligosaccharides, are the main products of photosynthesis (Bodelón et al., 2010). Collective evidence indicates that photosynthetic activity is inhibited during the development of drought stress, which may explain the decreasing soluble sugar accumulation under drought stress, especially severe drought, compared with the control condition. DA8 was the only variety, which did not decrease leaf soluble sugar under 2015 moderate drought. DA8 also expressed a significant increase in sheath soluble sugar content under 2015 moderate drought in comparison with control condition. Under moderate and severe drought conditions in 2015, DA8 and Thierno Bande always expressed higher sheath soluble sugar contents than the other varieties. These results indicated that the accumulation and degradation of soluble sugar under drought stress conditions depends not only on the severity of drought stress, but also on the characteristics of the different varieties. Previous studies have suggested that soluble sugar has a role as an osmoprotectant, regulating osmotic adjustment, providing membrane protection, and scavenging toxic reactive oxygen species under various types of stress (Ahmad & Satyawati, 2008). In our previous experiment (Dien, Mochizuki, et al., 2017), DA8 and Thierno Bande were expressed as drought-tolerant varieties, and Malagkit Pirurutong was considered to be a drought-sensitive variety. Thus, greater soluble sugar accumulation in DA8 and Thierno Bande may be contributed to drought tolerance in these varieties compared with other varieties.

Starch content in leaves and sheaths of varieties was significantly decreased under moderate drought compared to control condition, except for DA8 and Malagkit Pirurutong in 2015, and Kinandang Patong in 2017. Severe drought significantly decreased starch content in leaves and sheaths of varieties, except for DA8 and Kinandang Patong in 2015 (Figure 7). The tendency of starch degradation under drought stress in this study approved previous studies, which suggested that starch is degraded under drought condition to provide energy and carbon when photosynthesis activity is restricted (Siaut et al., 2011). Surprisingly, leaf starch content of DA8 and Malagkit Pirurutong significantly increased under 2015 moderate drought in comparison with control condition. In a previous experiment, Xu et al. (2015) found that stem starch content of different rice varieties was differentially changed under drought stress condition. The reason for higher accumulation of starch content under drought stress is not clear. After re-watering from drought treatment, starch content in leaves and sheaths of varieties generally still remained lower than those under control conditions.
condition. This suggested that starch accumulation was severely affected by drought stress and difficult to recover. DA8 generally expressed higher starch content than other varieties at 1 day re-watering after moderate drought (Figure 7(b,d)). It indicated that DA8 had higher recovery ability in starch accumulation than other varieties.

Overall, drought stress decreased soluble sugar and starch content of rice varieties in comparison with control condition. However, there were fluctuations in soluble sugar and starch content under control condition. In addition, after re-watering, the content of soluble sugar and starch generally remained significantly lower than those in control condition. This suggested that there was no direct relationship between plant water content and contents of soluble sugar, starch in rice varieties. Metabolism of soluble sugar and starch in rice was affected by both drought stress condition and characteristics of varieties.

5. Conclusion

Our study found a large variation in responses of different rice varieties under drought stresses and subsequent recovery. Under drought stress conditions, water content in leaves and sheaths of varieties dramatically decreased compared with the control condition. This study revealed that water in plants was highly stored in sheaths than in leaves under control or moderate drought. However, distribution of water in plant was higher in leaves than in sheaths under severe drought. This might be a response of plant in order to protect their leaves from devastation by dehydration stress. To protect against severe damage of drought stress, rice plants highly accumulated proline as an osmoprotectant. Under the same stress condition, accumulation of proline is higher in leaves than in sheaths, and drought tolerant varieties (DA8 and Thierno Bande) accumulated higher proline than susceptible ones (especially Malagkit Pirurutong). Re-watering after drought stress quickly degraded proline content in leaves and sheaths of all varieties. At 7 days after re-watering from moderate and severe drought, proline content of all varieties fell down to the same level of control condition. The ability of proline accumulation can be used as a useful indicator for drought tolerant potential of rice varieties.

Soluble sugar and starch contents in leaves and sheaths of varieties were significantly decreased under drought stress with some exceptions. In comparison with susceptible varieties, soluble sugar and starch contents of tolerant varieties were less negatively affected by drought stresses. While accumulation and degradation of proline were mainly affected by environmental conditions, metabolism of soluble sugar and starch in rice varieties were also affected by growing stages. This indicated that researchers should carefully consider about growing stages of plant while studying about carbohydrate metabolism under drought condition.

Disclosure statement

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