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Proline-related gene expression contribute to physiological changes of East Nusa Tenggara (Indonesia) local rice cultivars during drought stress

YUSTINA CAROLINA FEBRIANTI SALSINHA[1,2], SITI NURBAITI[1], ALFINO SEBASTIAN[2], DIDIK INDRADEWA[3], YEKTI ASIH PURWESTRI[1,2], DIAH RACHMAWATI[1,♥]

[1]Department of Tropical Biology, Faculty of Biology, Universitas Gadjah Mada. Jl. Teknika Selatan, Sekip Utara, Sleman 55281, Yogyakarta, Indonesia. Tel./fax.: +62 274-58 ♥email: drachmawati@ugm.ac.id
[2]Research Center for Biotechnology, Universitas Gadjah Mada. Jl. Teknika Utara, Barek, Sleman 55281, Yogyakarta, Indonesia
[3]Department of Agronomy, Faculty of Agriculture, Universitas Gadjah Mada. Jl. Flora, Bulaksumur, Sleman 55281, Yogyakarta, Indonesia

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Abstract. *Salsinha YCF, Nurbaiti S, Sebastian A, Indradewa D, Purwestri YA, Rachmawati D. 2022. Proline-related gene expression contribute to physiological changes of East Nusa Tenggara (Indonesia) local rice cultivars during drought stress. Biodiversitas 23: 3573-3583.* An osmoregulation response is one of the mechanisms of adaptation to drought stress. Concerning the synthesis and catabolism of proline as an osmoprotectant, this research aims to study the involvement of proline in regulating physiological change in local rice cultivars from Nusa Tenggara Timur (NTT), Indonesia. Five rice cultivars consisting of Ciherang (drought susceptible) and Situ Bagendit (drought tolerant) and three local rice cultivars, namely Gogo Jak (GJ), Kisol Manggarai (KM), and Boawae Seratus Malam (BSM) were used. Drought treatments were carried out using the fraction of transpirable soil water (FTSW) with control (FTSW 1) and severe drought (FTSW 0.2). The upregulation of *OsP5CS* and *OsP5CR* as proline synthesis genes in GJ leaves led to higher proline levels. At the KM cultivar, there was an upregulation of *OsPRODH* related to proline catabolism. Furthermore, *OsNHX1*, responsible for proline transport, was higher in the GJ cultivar. The presence of proline also correlated with physiological adaptations. Proline accumulation due to the activation of proline-related gene expression caused less physiological changes and antioxidant activity reduction and has led to maximum water absorption during a severe drought in the BSM cultivar. The analysis showed non-significant decreased levels of chlorophyll-a and increased levels of chlorophyll-b, carotenoids, and anthocyanins of drought-tolerant cultivars lead to higher leaf number, plant height, and tiller number under drought. Meanwhile, varied activity levels of superoxide dismutase (SOD), ascorbate peroxidase (APX), and catalase (CAT) were observed in line with the slight increase in H_2O_2 and malonaldehyde (MDA) levels during severe drought.

Keywords: Drought, gene expression, local rice, osmoregulation, proline

Abbreviations: APX: ascorbate peroxidase, BSM: Boawae Seratus Malam, CAT: catalase, GJ: Gogo Jak, H_2O_2: hydrogen peroxide, KM: Kisol Manggarai, MDA: malonaldehyde, NTT: Nusa Tenggara Timur, SOD: superoxide dismutase

INTRODUCTION

Drought stress is one of the inevitable stresses limiting crop production worldwide. Plant responses to drought could vary among species depending on the plants' adaptability to stress conditions. According to Stewart (Stewart 2002), drought-tolerant plants can organize the ultrastructure part or carry out metabolic adaptation in further extensive water loss conditions. One of the metabolic adaptations to drought occurs through osmoprotectant accumulation. The accumulation in the forms of proline, sucrose, glycine-betaine, and the other compound in the cytoplasm act as an osmotic adjustment, increasing the rate of water absorbed in drought environment (Usman et al. 2013; Maisura et al. 2014).

Proline is an amino acid most frequently analyzed for its role as an osmoprotectant in drought tolerance plants. Under severe drought, proline concentrations increased higher than the other amino acids. This effect was used as a marker for selecting the highly adaptable plant in avoiding cell damage caused by drought stress (Fahramand et al. 2014). The high solubility of proline in water makes it more capable of adjusting the osmotic potential of cells (Ashraf et al. 2018). Research conducted by De Carvalho (De Carvalho et al. 2013) and Shinde (Shinde et al. 2016) reveal that proline plays a crucial role as a potential component of cellular redox buffer and maintains the ratio of $NADP^+$ to NADPH. The degradation of proline molecules also provides energy for regrowth in a period of rehydration (Szepesi and Szollosi 2018). In some cases, proline acts as a signal-molecule of the plant exposed to abiotic stress conditions (Hossain et al. 2014). The proline level changes were also observed in rice (Bunnag and Pongthai 2013; Lum et al. 2014; Maisura et al. 2014).

During dehydration, proline accumulation occurs through a series of regulations. Several enzymes played a role in the proline regulation, including P5CR (Δ 1-

Pyrroline- 5- carboxylate reductase) and P5CS (Δ 1-Pyrroline- 5-carboxylate synthetase), responsible for proline synthesis from glutamate, and OAT (ornithine aminotransferase) responsible for synthesizing proline from ornithine. Meanwhile, proline catabolism is catalyzed by PRODH (proline dehydrogenase) and P5CDH (Δ 1-Pyrroline-5-carboxylate dehydrogenase) (Sharma et al. 2012; Fahramand et al. 2014). Previous studies have shown that photosynthetic organs have a higher level of gene expression responsible for proline synthesis (*P5CS* and *P5CR*), while the catabolism gene (*PRODH*) in these organs is suppressed. In contrast, proline breakdown occurs significantly in root organs, particularly in the meristematic zone (Sharma et al. 2012).

Apart from osmoprotectants, the antioxidant activity also increased in plants associated with reactive oxygen species (ROS) scavenging activity. In plants exposed to drought, high production and accumulation of hydrogen peroxide (H_2O_2) through oxidative activity encourages the accumulation of solutes in the form of α-tocopherols, phenolics, flavonoids, and proline, which function as non-enzymatic antioxidants that play a role in suppressing lipid-peroxidation along with SOD (superoxide dismutase), CAT (catalase) and APX (ascorbate peroxidase) activity during ROS scavenging (Rasheed et al. 2014; Ashraf et al. 2018).

Another response of a plant to dehydration also occurs through the regulation of cellular ion homeostasis. One of the homeostasis mechanisms occurs through regulations of the Na^+/H^+ (NHX) and H^+pyrophosphatase (H+Ppase, VP) antiporters located in the tonoplast by pumping H^+ from the cytosol to the vacuole, which allows the transport of organic acids and sugar components to increase the water potential of cells thereby increasing the potential for water absorption into cells (Fukuda et al. 2004; Liu et al. 2010).

As one of the regions with the dominance of dry climates, Nusa Tenggara Timur (NTT) - Indonesia is rich in rice germplasm adapted to drought conditions (Salsinha et al. 2021a). This study elucidated the mechanism of drought tolerance in the local rice cultivars through physiological and molecular analysis of proline osmoregulation, antioxidant activities level, and other physiological characteristics and their osmoregulation. Therefore, this study aimed to investigate the osmoregulation of local NTT rice plants in the molecular gene expression related to the synthesis and catabolism of proline and their relationship to physiological changes during severe drought stress.

MATERIALS AND METHODS

Plant materials

Five rice cultivars were used in this study, consisting of three local cultivars, namely Gogo Jak (GJ) and Boawae Seratus Malam (BSM) as drought-tolerant local cultivars and Kisol Manggarai (KM) as drought-susceptible local cultivar (Salsinha et al. 2021a, 2021c) with two control cultivars: Ciherang (drought-susceptible cultivar), Situ Bagendit (drought-tolerant cultivar).

Experimental site

This study was caried out at the Faculty of Biology Universitas Gadjah Mada, Indonesia, in the greenhouse of the Sawitsari Research Station with an altitude of 7°45′22″ S, 110°23′18″ E with an average rainfall of 250-350 mm, temperatures ranging between 24ºC to 34ºC and the sunlight intensity ranging from approximately 5,500 lx to 11,000 lx during the day.

Experimental design and treatment

Randomized-complete blocked design was used to arrange this research containing two factors: drought treatments (two levels of FTSWs) and cultivars (five cultivars) with three replications each. The 21 days old individual seedling was transferred into a pot consisting of 1 kg growing media (compost and soil with a ratio of 1:3, respectively) (Salsinha et al. 2020). Drought treatments were carried out using the fraction of transpirable soil water (FTSW) method with the control treatment (FTSW 1) and severe drought stress (FTSW 0.2) after seven days of acclimatization (Serraj et al. 2008). Before the treatment was started, each plant's total transpirable soil water (TTSW) was previously obtained by treating the plant with drought until it reached the permanent wilting point. Firstly, after reaching 100% field capacity, each cultivar's pot and plant weight were recorded as the initial weight (P_0). Then, the pot and plant were left for several days until they reached the permanent wilting point (showed by the stable weight), and the pot and plant weight were recorded again as P_i (weight at the permanent wilting point), and the TTSW of each cultivar was calculated with the formula:

$$TTSW = P_0\,(g) - P_i\,(g) \quad (1)$$

To maintain the stability of each FTSW (1 or 0.2), the pot and plant weight at any given time (Pt) and the amount of water (Wt) were kept stable along the treatment periods. The P_t and the W_t values were then calculated according to formulas (2) and (3), respectively, as follows:

$$P_t\,(g) = P_0 - (TTSW - W_t)$$
$$W_t\,(mL) = FTSW \times TTSW$$

Samples for molecular and physiological analysis were collected from plants 46 days after planting.

Molecular gene expression

The total RNA was isolated from leaves and roots of rice separately according to the protocol of FavorPrep™ Plant RNA Mini Kit 001-1 (Ping Tung Agricultural Biotechnology Park, Taiwan). Samples of about 100 mg each were collected from leaf and homogenized with FARB buffer containing ß-mercaptoethanol. After 5 mins incubation, the supernatant was homogenized with 70% sterile alcohol. After several separations with a centrifuge, the column sample was washed with washing buffer 1 and washing buffer 2 containing 96% ethanol. Total RNA was eluted with nuclease-free water, and the RNA purity was determined qualitatively by electrophoresis and

quantitatively by using a nanodrop spectrophotometer (Bichrome Nanodrop).

RNA purification was done using the DNAse treatment with the DNAse I kit (Sigma Aldrich D5307–Germany). The protocol of Excel RT Kit II (SMOBIO Technology, Inc-Taiwan) was used to carry out first-strand cDNA synthesis with a total cDNA amount of 500 ng. Each sample's gene expression levels was analyzed using quantitative real-time PCR (qRT-PCR) according to the protocol provided by ExcelTaq 2X Q-PCR Master Mix (SYBR, no ROX) (TQ1110 SMOBIO Technology, Inc-Taiwan). The mixture containing 1 µL of cDNA template, 1 µL forward primer, 1 µL reverse primer (Table 1), 5 µL 2X Q-PCR Master Mix, and 2 µL of ddH_2O were homogenized. The mixture containing the cDNA template was amplified in BioRad CFX96 qRT-PCR with the program consisting of template denaturation and enzyme activation stage at 95°C for 2 mins, the denaturation stage at 94°C for 30 seconds, annealing was done according to the Tm (melting temperature) of each target gene for 1 min and extension temperature of 72°C for 30 seconds, all in 39 cycles. The relative quantification of gene expression level in each sample was calculated after Duplo-Duplo experiments using the formula (Hong-zheng et al. 2017):

$$Relative\ expression = 2^{-\Delta\Delta Ct}$$

Where:

$$\Delta\Delta Ct = \Delta Ct\ unknown\ sample - \Delta Ct\ calibrators$$

$$\Delta Ct\ unknown\ sample = Ct\ internal\ reference\ gene - Ct\ target\ gene$$

$$\Delta Ct\ calibrator = Ct\ reference\ gene\ in\ ref\ sample - Ct\ target\ gene\ in\ ref\ sample$$

Leaf physiological characters

The anthocyanin content was measured spectrophotometrically according to Lotkowska (Lotkowska et al. 2015). Third leaf samples of about 0.25 mg were homogenized in 1 mL of extraction buffer containing 37% of HCl, 1-propanol, and bidest and incubated for 2 hours at room temperature after 5 mins incubation at 95°C. The absorbances of supernatants were measured at room temperature with multiwavelength at 535 nm, 620 nm, and 720 nm using a spectrophotometer (GENESYS 10 UV Scanning, Thermo Fisher Scientific). The Chlorophyll (Chl a and Chl b) and carotenoid content were analyzed from 0.03 g third leaf samples. Samples were homogenized in 80% cold acetone, and the absorbance of supernatants was measured using the multiwavelength of 470 nm, 645 nm, and 664 nm, according to Harborne (Harborne 1984).

The Proline level of samples was measured according to the Bates method (Bates et al. 1973). Leaf samples of about 0.25 g were homogenized in 5 mL of 3% sulfosalicylic acid, and 1 mL of filtered supernatant was reacted with 1 mL of CH_3COOH and 1 mL of ninhydrin acid, then incubated at 94°C for 1 hour and chilled in an icebox. Two mL of toluene were added to separate the proline from the organic phase. The absorbances were measured at 520 nm, and the proline content was determined by comparing the results with a standard curve of proline.

Antioxidant assays

The antioxidant activity of samples was measured through the activities of SOD, APX, CAT, and H_2O_2 contents. For enzymatic antioxidant activities assays, enzyme extraction was carried out by extracting 0.2 g of third leaf samples in 2 mL 50 mM potassium phosphate buffer (pH 7) containing 1 mM EDTA and 1% PVP (b/v). Supernatants were kept on ice during the measurement (Elevarthi and Martin 2010).

The SOD activity was measured by reacting enzyme samples with a Tris-HCl buffer (pH 8.2) buffer and 0.1 mM EDTA. The ddH_2O and 4.5 mmol L^{-1} pyrogallol were added to start the reactions. The absorbance was measured spectrophotometrically at 325 nm, and the enzyme-specific activity was expressed by 50% inhibition of pyrogallol autoxidation activity within 3 mins (Marklund and Marklund 1974).

CAT activity was measured based on the protocol by Elevarthi and Martin (Elevarthi and Martin 2010) with the reaction solution containing 200 µL of the extract mixed with 2.2 mL of 50 mM sodium phosphate buffer (pH 7) and 1 mL of 3% H_2O_2 solution. After 1 min incubation, absorbance was measured at 240 nm for 2 mins with a spectrophotometer, and specific enzyme activity was expressed from H_2O_2 decreasing per minute per mg of protein.

Table 1. Primers for real-time quantitative PCR of proline-related genes and Actin1 (Salsinha, 2021c)

Gene	Accession no.	Forward primer	Reverse primer
OsNHX1	AB021878.1	GCTAGATTTGAGCGGCATTC	GTCACTGGCAAACTCCCATT
OsP5CR	XM_015755311.2	GCACTTGGTCTTGCATCTCA	TCGCACATCCAATGGACTAA
OsPRoDH	XM_015757226.2	CGTACCTCATCAGACGAGCA	GATCGCTTCACTCCCAAGTC
OsP5CS	AY574031.1	TGCGAGCAGGTTAAGGAACT	GGCACAAGCCTTTCCATCTA
Actin1	EU650177	AGCCACACTGTCCCCATCTA	TCCCTCACAATTTCCCGCTC

Determination of APX activity was based on the method of Elevarthi and Martin (Elevarthi and Martin 2010) by reacting 100 µL extract with buffer containing 1.3 mL of 0.1 mM EDTA in a 0.05 mM sodium phosphate buffer, 0.8 mL 0.05 mM ascorbic acid, and 1.5 mL ddH_2O. After 1 min incubation, about 0.8 mL of 3% (v/v) H_2O_2 solution was added, and the absorbance was measured using a spectrophotometer at 290 nm.

The malondialdehyde (MDA) assessment was performed according to the method described by Hodges (Hodges et al. 1999). First, the third leaf sample was homogenized with 3 mL 0.1% trichloroacetic acid (TCA) and centrifuged at 8000 rpm for 15 mins. Next, about 1 mL supernatant was placed into microcentrifuge and reacted with 4 mL 10% TCA containing 0.65% thiobarbituric acid (TBA) and incubated at 90°C for 30 min. Finally, samples were centrifuged at 4000 rpm for 10 mins and measured at 440, 532, and 600 nm.

H_2O_2 content was measured on leaves by homogenizing 0.25 g of leaves in 2.5 mL 0.1% TCA (Bouazizi et al. 2007). About 0.5 mL supernatants were taken and reacted with 50% TCA, 10 mM ferrous ammonium sulfate, and 2.5 M potassium thiocyanate (KSCN). The absorbance was determined at 390 nm, and H_2O_2 content was determined using a standard curve of H_2O_2.

Growth parameters

The growth parameters observed were plant height, number of leaves, number of tillers, and root length. Plant height was measured from the stem base to the tip of the longest leaf. The number of leaves was calculated from each clump of plants. It includes the accumulated number of the youngest leaves that have started to stretch to the oldest brown leaves. The number of tillers counted as the number of clumped parts of the plant that grew above the ground and had leaves and shoots. Finally, root length was measured from the base to the tip of the longest root.

Statistical analysis

The significant differences in the data between all treatments in each parameter (chlorophyll content, carotenoid content, anthocyanins content, enzymes activities, H_2O_2, and MDA content) were tested and obtained from the ANOVA test, while the molecular expression level of proline-related genes was analyzed using T-test. Both Anova and T-test were followed by the Duncan range test with an interval confidence level of 95%. The correlation among parameters was analyzed using Pearson Correlation Test performed using SPSS Software (IBM-SPSS Ver 25.00.US).

RESULTS AND DISCUSSION

Proline related genes expression

One metabolic process that ensures water conservation in cells occurs through proline metabolic regulation as an osmoprotectant during drought. Figure 1 shows the difference based on the T-test followed by Duncan's test at the 95% confidence level in the FTSW 0.2 treatments in all gene expression. The data showed a significant interaction effect between FTSW and cultivars on the activity of genes related to proline metabolism in leaf tissues. Drought tolerant plants undergo an osmotic adjustment reflected by an increase in the activity of enzymes in proline anabolism which is supported by up-regulation of the associated genes.

Most proline biosynthesis occurs in the leaves (Ashraf et al. 2018). Based on the data (Figure 1A), the *OsP5CS* expression level was upregulated in all cultivars but higher in the BSM and GJ, which were significantly different ($p<0.05$) from KM during severe drought treatment. Figure 2b shows the activity of the *OsP5CR* gene that functions to convert P5C to proline. Although most of the cultivars showed upregulation of this gene, the expression level was higher in the leaves of GJ and KM cultivars. Both GJ and KM showed no differences from each other ($p>0.05$), but both were significantly different from the BSM cultivar ($p<0.05$). Based on the data (Figure 1B), the *OsP5CR* was upregulated in both the GJ and KM cultivars but was downregulated in the BSM cultivar.

The increased activity of *OsP5CS* and *OsP5CR* leads to the accumulation of proline during drought. Proline catabolism is regulated by *OsProDH*. Based on the data (Figure 1C), the expression levels of *OsProDH* in the leaves show no significant differences ($p>0.05$) among all cultivars. Furthermore, the GJ, KM, and BSM cultivars were not significantly different in the expression levels of *OsProDH*, causing similar proline catabolism levels between these cultivars.

Based on Figure 1D, the upregulation of *the OsNHX1* level responsible for the activation of the H^+ PPase antiporter showed no significant differences between KM and GJ cultivars. However, the cultivar of BSM showed a lower expression level of the *OsNHX1*. This adaptability through antiporter and H^+ PPase regulation allows the transport of osmolites, especially proline, during the dry phase (Fukuda et al. 2004; De Carvalho et al. 2013). The study concluded that proline regulation occurs through the activation of *OsP5CS*, *OsP5CR*, *OsProDH*, and *OsNHX1* (Salsinha 2021c).

During drought, higher proline synthesis requires a transport mechanism (one of which is antiport and H^+ PPase) that supports proline function in osmoregulation. This process can be seen in the accumulation of proline as the main osmoprotectant and MDA level as the intermediate compound during lipid peroxidation. Therefore, plant with high expression activity of the *OsP5CS*, *OsP5CR*, and *OsNHX1* (Figure 1.A, B, D), tends to accumulate higher proline content (Figure 2.A) and lower level of MDA (Figure 2.B), thereby reducing the level of the cell damage caused by lipid peroxidase and other ROS.

Physiological changes for local rice cultivars

The increase in the proline accumulation will be proportional to the drought stress given to the rice plant (Raye et al. 2018). The data (Figure 2.A) showed a significant increase in proline levels in several rice cultivars under severe drought compared to control. Based on the data (Figure 2A), it can be seen that during severe

drought stress (FTSW 0.2), GJ and BSM cultivars ($p>0.05$ between both cultivars) tended to have higher ($p<0.05$) proline content compared with KM. On the other hand, the KM cultivar showed no significant differences with CH as a drought-susceptible control.

Figure 2.B shows the physiological response of an intermediate compound malonaldehyde (MDA) produced during lipid peroxidation. During severe drought conditions, membrane damage can be observed from the levels of MDA formed. The effect of proline accumulation led to the decrease of MDA during drought. Plants with higher proline tend to show lower content of MDA. The data (Figure 2B) show that all cultivars' MDA levels were lower during the control condition (FTSW 1). During severe drought, all cultivars showed a high level of MDA with no significant differences ($p>0.05$) among KM, GJ and CH cultivars. On the contrary, the SB and BSM cultivars produced lower and no significant differences ($p<0.05$) MDA levels.

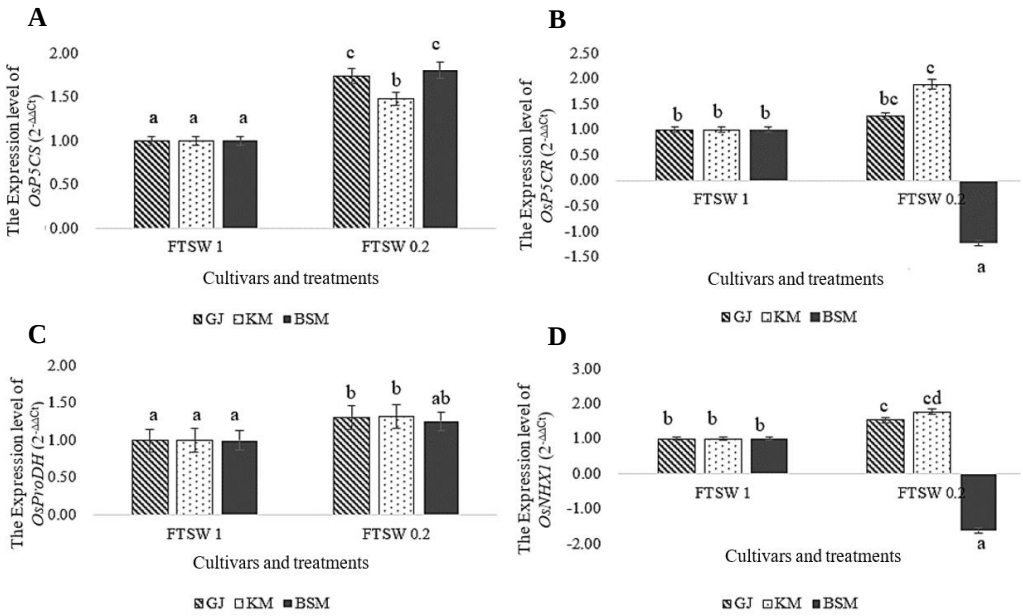

Figure 1. The relative expression levels of functional genes. A. *OsP5CS*, B. *OsP5CR*, C. *OsProDH*, D. *OsNHX1* against Actin1 in the leaf of local rice cultivars NTT: BSM (Boawae Seratus Malam), GJ (Gogo Jak) and KM (Kisol Manggarai) with treatment FTSW 1 and FTSW 0.2. The data shown in the form of the mean (n=3) with the same letter in each different gene showed no difference based on Duncan's test at the 95% confidence level. The data showed an interaction between FTSW and cultivars on the observed variables

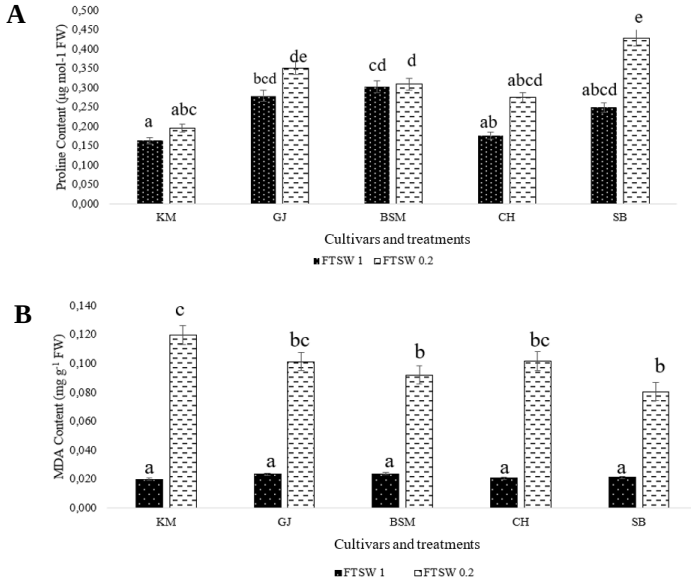

Figure 2. The content of proline and malonaldehyde (MDA) in leaves of KM (Kisol Manggarai), GJ (Gogo Jak), BSM (Boawae Seratus Malam), CH (Ciherang), and Situ Bagendit (SB). A. proline content, B. MDA in FTSW 1 (control) and FTSW 0.2 (severe drought) during the vegetative phase. Data shown as mean (n=3) with the same letter in each parameter shows no significant difference based on the Duncan test with a 95% confidence level

Based on Figure 3, during drought stress, there were increased SOD and APX activity levels in KM, BSM, and CH cultivars, while GJ and SB experienced a decreased level of SOD and APX activities. In contrast to SOD and APX, CAT activity decreased, which was in line with the increase in stress levels. The drought-tolerant SB and BSM cultivars showed higher SOD and CAT activity but a slightly lower APX activity and H_2O_2 content when exposed to drought. Conversely, the drought-susceptible cultivars (CH and KM) tended to show lower CAT activity but higher H_2O_2 content during severe drought, even though they showed high SOD and APX activity.

Figure 4 showed differences in photosynthetic characters as reflected by the levels of photosynthetic pigments (chlorophyll a and b), carotenoids, and anthocyanin pigments under control (FTSW 1) and drought stress (FTSW 0.2) of the five cultivars tested. Based on data (Figure 4 A, B), most of the cultivars experienced a significant decrease in chlorophyll a content but an increase in chlorophyll b and, hence, pigmented contents during FTSW 0.2 ($p<0.05$). In addition, the data in Figure 4 shows the BSM cultivar with significantly higher chlorophyll b content compared to other cultivars ($p<0.05$) during severe drought stress.

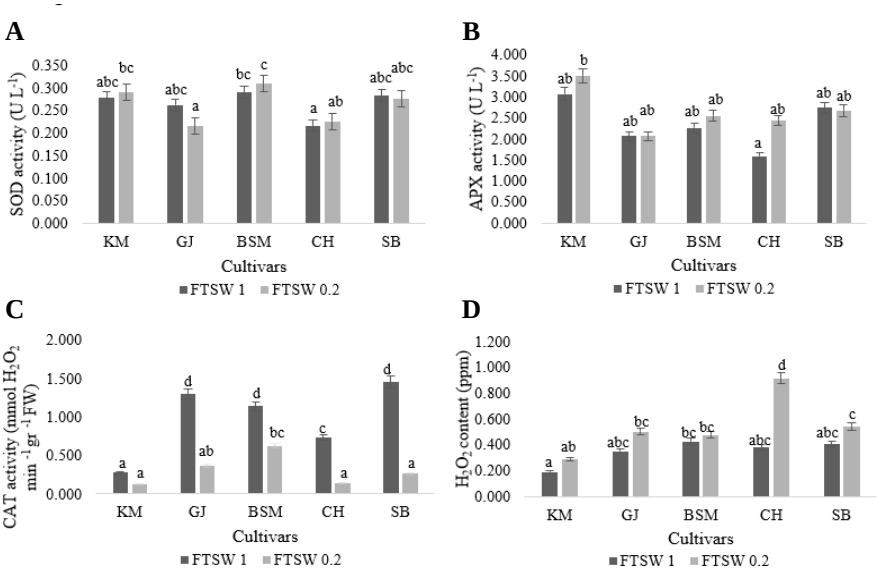

Figure 3. The activity of antioxidant enzymes across cultivars and drought conditions. Enzyme activity and H_2O_2 content in leaves of KM (Kisol Manggarai), GJ (Gogo Jak), BSM (Boawae Seratus Malam), CH (Ciherang), and Situ Bagendit (SB). A. SOD, B. APX, C. CAT, D. H_2O_2 in FTSW 1 (control) and FTSW 0.2 (severe drought) during the vegetative phase. Data shown as mean (n=3) with the same letter for each parameter shows no significant difference based on the Duncan test with a 95% confidence level

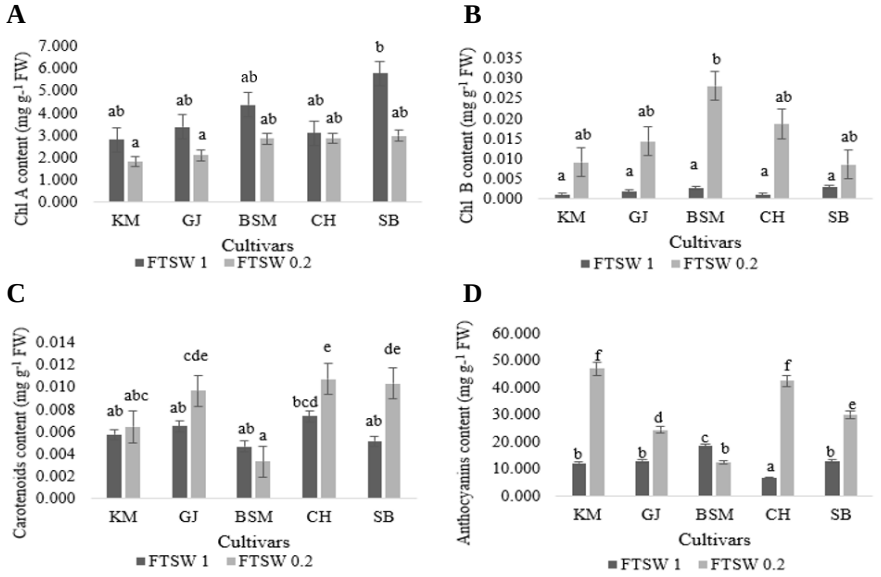

Figure 4. The chlorophyll, carotenoid, and anthocyanin content in leaves of KM (Kisol Manggarai), GJ (Gogo Jak), BSM (Boawae Seratus Malam), CH (Ciherang), and Situ Bagendit (SB). A. chlorophyll a, B. chlorophyll b, C. carotenoid, D. anthocyanins content in FTSW 1 (control) and FTSW 0.2 (severe drought) during the vegetative phase. Data shown as mean (n=3) with the same letter for each parameter shows no significant difference based on the Duncan test with a 95% confidence level

The data (Figures 4C and 4D) showed significant differences in carotenoid content among all cultivars during severe drought. Figure 4C specifically shows that the GJ and CH cultivar had higher carotenoid contents in severe drought, while the BSM cultivar showed a lower carotenoid level. Figure 4D shows higher anthocyanins content in KM and CH during a severe drought that was significantly different from other cultivars.

Morphological changes of local rice cultivars

The accumulation of physiological and metabolic changes is reflected in the growth phenotype characters. The data (Table 2) shows significant plant height, leaf number, tiller number, and root length differences during FTSW 1 and FTSW 0.2 in all cultivars. During FTSW 1 (control), the BSM cultivar tended to have a higher plant size than GJ and KM ($p<0.05$), and the plant height of BSM and Situ Bagendit was also significantly different. At the FTSW 0.2, BSM also showed a higher plant height than CH. The significant difference in response to FTSW 1 and FTSW 0.2 treatments in each cultivar indicated an interaction between cultivar and FTSW, especially in KM, GJ, and BSM cultivars. Leaf numbers were observed not significantly differed among cultivars during FTSW 0.2 except between BSM and CH. Tiller numbers (Table 2) were also observed to significantly differ between BSM and control but not significantly among other cultivars when exposed to drought. The root length of all roots significantly differed ($p<0.05$) with no interaction between cultivars. During drought, the lowest length is shown by CH, followed by BSM, GJ, SB, and KM cultivars, respectively.

Data (Table 2) also shows that drought stress significantly reduced growth parameters by triggering changes in the growth cycle and plant development from vegetative to reproductive phases. This is also caused by cell membrane damage during drought treatment (Farooq et al. 2012). The characteristics of plant height, number of tillers, and plant biomass depend on the activity of cell division, expansion, and differentiation (Farooq et al. 2012; Ai-hua et al. 2017). The changes in morphological characters are shown in Figure 5.

Table 2. Morphological characters of 3 local NTT rice cultivars and two check cultivars in response to decreased FTSW levels

Cultivar	Plant height (cm)		Leaf number	
	FTSW 1	FTSW 0.2	FTSW 1	FTSW 0.2
Kisol (KM)	85.50 ^{c}	66.83 ^{ab}	13.00 ^{bc}	9.00 ^{ab}
Gogo Jak (GJ)	88.17 ^{cd}	70.33 ^{b}	13.33 ^{bc}	9.00 ^{ab}
Boawae Seratus Malam (BSM)	93.67 ^{d}	71.00 ^{b}	12.00 ^{bc}	7.33 ^{a}
Ciherang (CH)	66.33 ^{ab}	59.00 ^{a}	15.00 ^{cd}	10.33 ^{ab}
Situ Bagendit (SB)	69.83 ^{b}	62.83 ^{ab}	17.67 ^{d}	13.00 ^{bc}
Cultivar	**Tiller number**		**Root length (cm)**	
	FTSW 1	FTSW 0.2	FTSW 1	FTSW 0.2
Kisol (KM)	3.00 ^{bc}	2.00 ^{ab}	38.22 ^{i}	34.36 ^{f}
Gogo Jak (GJ)	2.33 ^{abc}	2.00 ^{ab}	33.38 ^{e}	22.80 ^{c}
Boawae Seratus Malam (BSM)	2.33 ^{abc}	1.33 ^{a}	36.78 ^{h}	22.27 ^{b}
Ciherang (CH)	3.00 ^{bc}	2.33 ^{abc}	36.56 ^{g}	14.54 ^{a}
Situ Bagendit (SB)	3.33 ^{c}	2.67 ^{bc}	34.30 ^{f}	29.34 ^{d}

Note: The mean value (n=3) followed by the same letter in the same parameter showed no significant difference based on ANOVA and Duncan test ($p<0.05$) with a 95% confidence level. The level of treatment given was FTSW 1: control, FTSW 0.2: severe drought stress

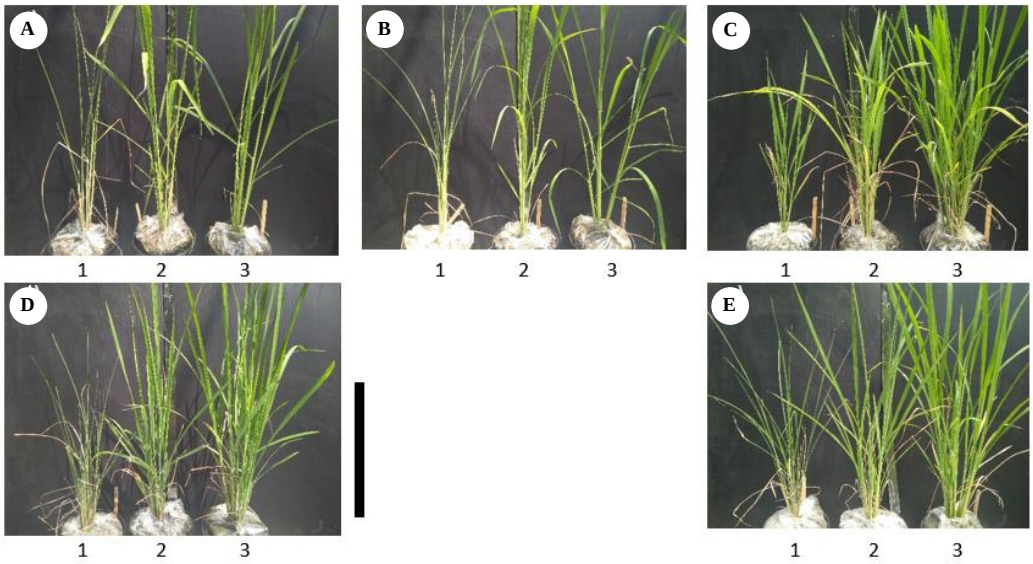

Figure 5. The morphological characters of: A. KM (Kisol Manggarai), B. GJ (Gogo Jak), C. BSM (Boawae Seratus Malam), D. CH (Ciherang), E. Situ Bagendit (SB) with 1) treated with FTSW 0.2 (severe drought); 2) treated with FTSW 0.5 (moderate drought) and 3) treated with FTSW 1 (control) during the vegetative phase. Bar: 30 cm

Discussion

Drought stress during the vegetative phase of rice causes a decrease in physiological function due to low water availability. Plant responses to these conditions occur through a series of adjustments, including transcriptomic regulation, functional gene regulation, and protein synthesis, leading to the osmotic and oxidative adjustments to support photosynthesis and other metabolic processes.

Proline regulation during severe drought stress is regulated through several processes. One of the steps of proline synthesis occurs through the activity of P5CS and P5CR enzymes responsible for proline anabolism and ProDH, which plays a role in proline catabolism (Lehmann et al. 2010; Guo et al. 2020). The continuity activity of these three enzymes is closely related to the activity of genes that regulate the synthesis of the enzymes involved and abiotic environmental factors. Upregulation of genes included in the synthesis of P5CS and P5CR leads to the accumulation of proline in the tissue, especially in the leaf organ.

In this study, the activity of *OsP5CS* was upregulated in all cultivars, with no significant differences between cultivars under severe drought. The activity of *OsP5CS* is also supported by *OsP5CR* upregulation in the GJ and KM cultivar under drought stress. The increased activity of *OsP5CR* and *OsP5CS* leads to the accumulation of proline during drought. During drought also, the expression levels of *OsProDH* in the GJ and KM cultivars were not significantly different while the BSM cultivar showed lower expression levels led to lower proline catabolism levels compared to other cultivars.

In several conditions, proline catabolism occurs to enhance energy conservation and promote plant growth. In this study, the high expression of *OsProDH* can be the strategy to maintain proline balance in cells, especially during recovery. Based on a study (Ashraf et al. 2018; Szepesi and Szollosi 2018), under-recovery conditions, the activity of the ProDH enzyme increases in line with the increase in water accumulation in cells, directing energy priority of cells to grow. Moreover, the reduction of proline contents correlated with the upregulation of the *OsProDH* gene that occurs in roots. Drought-tolerant plants with better osmoregulation ability are characterized by upregulation of proline anabolism genes (Ashraf et al. 2018; Guo et al. 2020). ProDH enzyme catalyzes proline into P5C (with an opposite reaction to P5CR enzyme (Shinde et al. 2016).

The transport process also affects the efficiency of anabolism and accumulation of proline. One gene that plays a role in ion transport in cells is *NHX1*. The *OsNHX1* gene plays a role in regulating Na^+ / H^+ antiporter in synergy with H^+ vacuolar pyrophosphatase (H^+ -PPase) to prevent damage due to Na^+ accumulation and H^+ pumping (Wang et al. 2001; Huang et al. 2014). The difference in the electrochemical potential gradient of H^+ between the cytoplasm and the vacuole can cause Na^+ / H^+ exchange which allows the transport of organic acids and osmolites into the cell (Fukuda et al. 2004; Liu et al. 2010; Wang et al. 2001).

In this study, the *OsNHX1* expression level in the KM cultivar was higher than in other cultivars. In line with its role, the upregulation of this gene allows plants to adapt to dehydration conditions, especially in conditions of low water or high salinity. Plants respond by regulating the osmotic pressure in the cell through an electrochemical gradient mechanism that causes osmolyte accumulation so that the water potential in the root cell is smaller than in the external environment.

Proline regulation during drought stress is differently regulated depending on the tolerance level of each cultivar. The optimal proline regulation in the drought stress phase is closely related to the protection of cells and tissues, ensuring that the metabolic process takes place within the range of cell homeostasis. Based on this study, proline regulation in plants occurs through the regulation of enzymes in proline biosynthetic and catabolic pathways.

The presence of proline is associated with the osmotic adjustment ability of the cell to reduce the rapid rate of water loss from the internal compartments of the cell (Farahani et al. 2013). Specifically, proline is synthesized when a plant experiences drought, although the biosynthetic and catabolic responses of each cultivar and species can differ. Previous research on *Zea mays* and *Triticum aestivum* (Vendruscoloa et al. 2007; Parida et al. 2016) showed that plants accumulate proline as a form of adaptation strategy to inhibit the drought-induced degradation effects through osmotic adjustment and its role as an antioxidant. In addition, based on a previous study (Vendruscoloa et al. 2007; Farahani et al. 2013), proline indirectly protects photosynthetic apparatus from thylakoid membrane damage due to dehydration during drought.

In this study, those related proline gene expression were observed in line with the accumulation of proline and the decreasing of MDA in all cultivars. The higher the activity of proline anabolism, the higher the proline content accumulated in cells, thus enhancing membrane protection against lipid peroxidation. Proline's role as an osmoprotectant also correlates with oxidative responses as the main responses of plants when coping with free radicals.

An oxidative response can occur through the activation of antioxidant enzyme reactions. For example, during severe drought stress, there was a large production of ROS (mainly through the O2-* formation in the light reactions of photosynthesis and photorespiration). ROS will disturb the lipid component of the cell membrane through lipid peroxidation leading to cell membrane damage and malfunction. Therefore, several enzymes were synthesized to perform ROS scavenging activity to prevent damage from this activity. These enzymes consisted of SOD, CAT, and APX. Those three enzymes that work in chain reactions converting ROS into a more stable compound harmless to cells include H_2O and O_2 (Refli et al. 2014; Fatikhasari and Rachmawati 2020).

Based on this study, the increase of proline levels in plants was in line with the higher activity of enzymatic antioxidants and the adjustment of pigment levels in the photosynthetic apparatus. The GJ and BSM cultivars

tended to have higher enzymatic antioxidant activities than KM (drought-susceptible). The effect of antioxidant activity and proline accumulation also ensures the plant's photosynthetic process.

Plants increase the production of antioxidants to minimize the detrimental effect of ROS and normalize metabolic activity during drought. SOD is specifically involved in a chain reaction that catalyzes the conversion of O_2^- to H_2O_2, which is then reduced to water by APX, GR, and CAT (Refli and Purwestri 2016; Nahar et al. 2018; Fatikhasari and Rachmawati 2020). Specifically, APX's catalytic activity utilizes ascorbate as an electron donor. Simultaneously, these enzymes work by alleviating elevated proline levels to minimize the damaging effects of ROS (Refli and Purwestri 2016). In plants with lower proline levels, oxidative antioxidants are responsible for protecting the cell apparatus, such as cell membranes and other organelles needed for photosynthesis and absorption of water.

In this study, the physiological process of photosynthesis was observed through the changes in photosynthetic pigments content including chlorophyll a, b and carotenoid. Chlorophyll content indirectly indicates the conditions of the photosynthetic compartment (Saito et al. 2018). Drought tolerant plants tend to show adjustment mechanisms that allow pigments and photosynthetic apparatus protection to slow down the rate of degradation and support the assimilation of plants in times of drought. Meanwhile, non-tolerant plants tended to experience greater reductions when exposed to drought conditions due to the absence of an adjustment mechanism.

The study results indicate that drought stress will lead to the accumulation of free radicals that will attack chlorophyll a and chlorophyll b, thereby reducing their quantity and quality in leaves. It will decrease the rate of photosynthesis and photoassimilate production. The adaptability of the BSM's photosynthetic apparatus to drought was considered tolerant with the lowest level of carotenoid and anthocyanin pigments. In addition, adjusting the photosynthetic process finally leads to better assimilation and biomass and productivity of plants.

Drought-resistant cultivars (BSM and GJ) can maintain a higher chlorophyll content than other cultivars. This plant adaptation mechanism supports sustainable photosynthesis processes and nutrient accumulation during drought stress. Therefore, a higher level of chlorophyll content indicates better photosynthetic adaptability in some cultivars. In particular, chlorophyll a is the main photosynthetic pigment in plants which absorbs light ranging from orange to red and violet to blue (the main energy needed in photosynthesis). Meanwhile, in contrast to chlorophyll a, chlorophyll b acts as an accessory pigment that helps absorb blue light energy under shading conditions. In addition, the leaves are considered the main location of photosynthesis. Therefore, decreased chlorophyll content of leaves will decrease the rate of photosynthesis, resulting in reduced formation of dissolved sugar compounds (sucrose, glucose, fructose) and starch (Rachmawati et al. 2019).

Apart from photosynthetic pigments, when plants are subjected to severe drought stress, they will cope by accumulating phenolics, flavonoids, anthocyanins, and proline in concentrations greater than normal conditions. Some of these secondary metabolites are associated with cell walls, while others are in free form in plant vacuoles. Carotenoids and anthocyanins play an important role as protective non-enzymatic components against oxidative damage caused by dehydration and excessive light. Specifically, carotenoids act as accessory pigments in the photosystem, which function to increase light absorption (in the range of 420-500 nm domain spectrum), protect the photosynthetic apparatus against ROS generated during abiotic stress, and maintain the stability of the lipid bilayer membrane against lipid peroxidation (Ramel et al. 2012; Havaux 2013). A higher level of carotenoids considers a greater protective function given to the photosynthetic apparatus of plants during severe drought stress (Havaux 2013).

In line with the role of carotenoids, anthocyanins in plants also play a protective role, especially in preventing ROS formation. The biosynthesis of this flavonoid compound increases in drought-susceptible plants compared to drought-tolerant plants due to the absence of a first-line defense in the form of other oxidative defenses during drought (Agati et al. 2012). The antioxidant activity that affects a plant's resistance to drought is reflected by the changes in cell structure with increasing stress levels. The root and stem organs are the most affected parts during dehydration.

According to the previous study (Salsinha et al. 2021b) plants experienced a reduction in root diameter and root density during drought. This process is due to the changes in the metabolic process when cells are exposed to drought stress. In this condition, plant adjustment occurs through the decrease in growth activity leads to the formation of smaller cells. Also, according to previous studies (Salsinha et al. 2021a, 2021b), changes in root diameter size are related to the ability of plants to increase the pressure that supports higher root penetration through the planting medium in response to a decrease in soil moisture content.

Drought-tolerant plants tend to maintain the cell structure's stability by ensuring a decrease in the rate of transpiration and loss of water through the leaves and by increasing the absorption of water through a denser root (Salsinha et al. 2020). The process of cell division is directly affected by the availability of water in the cell. Plants with better responses to drought will develop physiological mechanisms to prevent the rate of water loss. One is by accumulating proline as an osmoprotectant (Pandey and Shukla 2015; Dossa et al. 2017). Activating this compound encoded by the genes responsible for proline biosynthesis ensures the homeostatic processes inside the cell. This compound also plays a role in maintaining membrane integrity to support cell expansion and differentiation leading to higher biomass accumulation in plants with the highest proline accumulation.

The expression of *OsP5CS* leads to a positive correlation with a high accumulation of chlorophyll b, carotenoid, anthocyanin, and proline. In contrast, the

expression of *OsP5CR* leads to the high accumulation of anthocyanin and APX activity. In most samples, the expression of *OsProDH* was found to be opposite to the accumulation of chlorophyll a and catalase activity, while the expression of *OsNHX1* was found to be positively correlated with the accumulation of carotenoid, anthocyanin, and higher activity of APX. These correlations were analyzed based on the average of all the samples tested in this study. Meanwhile, each cultivar is undergoing a different process to cope with drought stress, leading to the morphological characteristics of plants under drought stress.

Based on this study, the effect of the gene expression responsible for proline regulation on plants' physiological and morphological performance can vary among cultivars. The cultivar of KM with high upregulation of *OsP5CR*, *OsProDH*, and *OsNHX* showed lower proline accumulation but high levels of MDA and H_2O_2. This induces the activity of the enzyme SOD and APX, which leads to a high antioxidation process to keep the photosynthetic apparatus stable. The stability of the photosynthetic apparatus is indicated by its pigment content. In KM cultivars**,** chlorophyll a, chlorophyll b, and carotenoid levels were low, while anthocyanin levels were high, and vice versa with the BSM cultivar. Photosynthetic stability accumulated in the morphological characters of plants. In KM, the highest number of leaves, number of tillers, and root length were than BSM and GJ cultivars, which showed the opposite performance. The presence of proline correlates with physiological adaptations towards drought stress. Proline accumulation due to the activation of proline-related gene expression caused less reduction in physiological changes and antioxidant activity and has led to maximum water absorption during a severe drought in BSM as the control cultivar. Better water absorption leads to better plant growth under drought conditions.

In this study, rice plants have different tolerance strategies for drought. In some cultivars, the regulation of proline accumulation significantly positively impacted physiological and morphological performance. Still, in the other cultivars, the regulation of proline did not necessarily increase the effect of plant tolerance when exposed to drought. For this reason, further studies on the regulatory effect of proline in a more comprehensive manner, both exogenous and endogenous proline, may be an opportunity in the future.

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