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Effect of Drought Stress on Proline Gene Expression, Enzyme Activity, and Physiological Responses in Thai Mulberry (*Morus* spp.)

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ABSTRACT

Mulberries are vital for the silk industry as the sole natural food for silkworms, but their quality and quantity can be greatly impacted by environmental factors, notably water shortages or droughts. In this study, the proline content and expression levels of the *Pyrroline-5-carboxylate reductase* (*P5CR*) gene in four recommended Thai mulberry varieties (Sakhonnakorn, Sakhonnakorn 85, Burirum 60, and Srisaket 84) and one standard drought tolerant variety (SRCM9809-34) were measured under drought stress. Additionally, physiological data and antioxidative enzymatic activities were also examined. The findings revealed that SRCM9809-34, a drought-tolerant variety, had the lowest proline content, followed by Sakhonnakorn 85, Burirum 60, while the highest proline content was observed in Srisaket 84. Although there was no correlation between the expression level of the *P5CR* gene and proline content, the overall trend in all varieties was the same: proline content increased after drought conditions. Regarding physiological responses, the wilting score showed similar results to proline content, with SRCM9809-34 having the lowest proline content and wilting score. Moreover, SRCM9809-34 exhibited the highest RWC, P_n and WUE values, as well as the lowest level of MDA and H_2O_2. Our results validated and indicated that SRCM9809-34 is a drought-tolerant variety. From this finding, among the four Thai mulberry varieties, Sakhonnakorn 85 exhibited the highest potential for drought tolerance, and this potential can be enhanced through crossbreeding with SRCM9809-34.

1. Introduction

Mulberry belongs to the family Moraceae, genus *Morus*. It is a widely cultivated crop plant in Asia, for example, India, China, Japan, Korea, and Thailand (Vijayan *et al.* 2011). Mulberry leaves are highly nutritious and the only natural food for silkworms (*Bombyx mori* L.) (Vijayan *et al.* 2018; Farahani *et al.* 2019). The quality of silk depends on the quality and quantity of mulberry leaves that feed the silkworm. Therefore, the selection of mulberry varieties is an important factor for farmers. In Thailand, mulberry is popularly cultivated in the northeastern region where the major silkworm industry is located. There are four recommended Thai varieties (Sakhonnakorn, Sakhonnakorn 85, Burirum 60, and Srisaket 84), none of which is tolerant to drought. SRCM9809-34, on the other hand, is a drought-tolerant variety that the Department of Sericulture, Thailand, has approved.

One of the major problems in mulberry cultivation is the shortage of water or drought. Many northeastern Thailand areas are affected by water shortages (Wipatayotin and Tangprasert 2019). Many physiological factors are affected by drought stress, including the inhibition of photosynthesis, a decline in chlorophyll content, and a decrease in the efficiency of photosystem II (PSII). Furthermore, plants exposed to water stress suffered from subsequent oxidative stress, leading to the production of reactive oxygen species (ROS) and resulting in enzyme inactivation, protein degradation, and cellular membrane disruption (Sonjaroon *et al.* 2016). Ultimately, water shortage will lead to a loss of crop production. Plants

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produce various osmoprotectants, including proline, glycine betaine, glycerol, and mannitol, when exposed to environmental stress such as drought and cold. An osmoprotective molecule protects plants from severe situations by acting as a small uncharged organic molecule (Dar *et al.* 2016).

The proline molecule is widely recognized as a major compatible solute in many plants under environmental stress. According to a comparative metabolome analysis, proline has been reported to primarily function in response to osmotic stress (Takahashi *et al.* 2020). Proline is an amino acid, an important part of living organisms and accumulates in eubacteria, protozoa, marine invertebrates, and plants under stress (Verbruggen and Hermans 2008). In stressful conditions, proline has played a significant role in minimizing adverse effects. Proline functions as a redox balancer for maintaining NADPH and $NADP^+$ levels, an osmoprotectant, and a signalling mechanism for maintaining feedback control in proline biosynthesis pathways (Szabados and Savoure 2010). The accumulation of proline under drought stress has been reported in many studies, for example, in wheat (Vendruscolo *et al.* 2007), petunia (Yamada *et al.* 2005), and peanut (Solanki and Sarangi 2014).

In plants, proline is derived from glutamic acid. It is produced by enzymes *Pyrroline-5-carboxylate synthase* (*P5CS*) and *Pyrroline-5-carboxylate reductase* (*P5CR*) through their proline biosynthesis pathway. The overexpression of *P5CS* or *P5CR* genes is reported to enhance plant stress tolerance; for example, the overexpression of the *P5CS* gene in switchgrass under salinity stress (Guan *et al.* 2019), tobacco under drought stress (Zarei *et al.* 2012) and *Lilium regale P5CS* (*LrP5CS*) in *Arabidopsis* under drought and salt stress (Wei *et al.* 2016). A similar phenomenon has been observed in sweet potatoes under salt stress (Liu *et al.* 2014) and in *Triticum aestivum* (*TaP5CR*) in *Arabidopsis* under various osmotic stresses (Ma *et al.* 2008). In this study, we examined proline content and expression level of the *P5CR* gene in Thai mulberries under drought stress. Additionally, antioxidative peroxidase enzyme activity and physiological data were measured. The information obtained from this study can be used for the selection of Thai mulberry varieties in the breeding programs in the future.

2. Materials and Methods

2.1. Plant Materials and Experimental Design

A total of five mulberry varieties were used in the study: four recommended Thai varieties (Sakhonnakorn, Sakhonnakorn 85, Burirum 60, and Srisaket 84), which are not tolerant to drought and one drought-tolerant variety (SRCM9809-34). The mulberries were grown in the flowerpot at the Advanced Plant Production with artificial light, located at the Kasetsart Agricultural and Agro-Industrial Product Improvement Institute (KAPI), Kasetsart University, Thailand. Five-month-old plants were used in a completely randomized design (CRD) with five replicates. The experiment consisted of two groups for each variety: a control group that received regular watering and a drought treatment group without irrigation. Leaf samples were collected, and physiological measurements and enzymatic activities were conducted at 0, 5, 6, and 7 days after stopping watering because the rate of photosynthesis, denoted as P_n, had decreased to 50% of its initial value on the fifth day after watering was stopped.

2.2. Proline Content and Gene Expression Analysis by Real-Time Quantitative PCR

Mulberry leaf samples weighing 0.1 grams were homogenized in 3% (w/v) aqueous sulfosalicylic acid and centrifuged at 10,000 xg for 20 min. After that, the supernatant was transferred to a new tube, mixed with 1 ml acetic ninhydrin and 1 ml glacial acetic acid, and incubated at 100°C for 30 min. 2 ml toluene was added after the reaction was stopped on ice. The aqueous phase was used to measure the absorbance at 520 nanometer using toluene as a blank. The proline concentration was estimated using a standard curve (Bates *et al.* 1973).

Total RNA was extracted from pooled leaf samples using a Total RNA extraction kit (Vivantis, Selangor Darul Ehsan, Malaysia) and treated with RNase-free DNase I to remove contaminated DNA. For cDNA synthesis, total RNA was reverse transcribed using oligo (dT) primers and ImProm-II reverse transcriptase (Promega, Madison, WI, USA). The cDNA was diluted to a 1:20 dilution and used as a template for qRT-PCR. Real-time PCR reaction of the *P5CR* gene was performed with the KAPA SYBR® FAST qPCR Master Mix (Kapa Biosystems, Wilmington, MA, USA) (forward primer 5'-AGGAGTTGTCCGATCCGGAA-3' and reverse primer 5'-CTTGGAGAGGACTCTGACGCC-3' with product size 111 bp). Actin was used as an internal control (forward primer 5'-GAGCAAGGAAATTTCTGCCC-3' and reverse primer 5'-TACTCCGACTTTGCGATCCAC-3' with product size 248 bp). Gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method after normalization with the actin gene. Gene expression value was compared between the control value and the treatment value of the same day.

2.3. Measurement of Wilting Scores

A wilting score ranging from 0–4 was recorded. Healthy plants, with leaves showing no signs of

wilting, were scored as 0. Plants on the verge of wilting or with some initial wilting leaves were scored as 1. Plants showing signs of wilting and starting to have yellow leaves were scored as 2. Plants that started wilting and had dried and brown leaves were scored as 3. Plants that continued to wilt, with most leaves turning brown and the whole plant dead, were scored as 4.

2.4. Relative Leaf Water Content (RWC) and Chlorophyll Content

The fresh weight (FW) of mulberry leaves was measured immediately after harvesting. After the FW measurement, leaves were soaked in distilled water for 24 hours and used for the total weight (TW) measurement. After that, leaves were dried at 65°C for 48 hours and used for the dry weight (DW) measurement. The relative leaf water content (RWC) was calculated according to the formula: [(FW − DW)/(TW − DW)] × 100% (Guan *et al.* 2018). In addition, leaf chlorophyll content was measured with a SPAD-502plus chlorophyll meter at 650 nm and 940 nm (Konica Minolta, Tokyo, Japan) (Ling *et al.* 2011).

2.5. Gas Exchange Rate and Chlorophyll Fluorescence Measurement

The net photosynthetic rate (P_n), stomatal conductance (g_s), and water use efficiency (WUE) were measured between 09.00–11.00 a.m. local time with an artificially saturating photon flux density at 900 µmol $m^{-2}s^{-1}$ using a portable photosynthesis system LI-6400XT (LI-COR, Lincoln, NE, USA). WUE was calculated as the ratio of P_n/E, where E represents the transpiration rate (Dos Santos *et al.* 2017). The photosynthetic measurements were conducted at a CO_2 concentration of 400 mol mol^{-1}, a relative humidity of 50-60% and a temperature of 30°C. Chlorophyll fluorescence was measured on leaves using a modulated chlorophyll fluorometer (Minipam-II, Walz, Effeltrich, Germany). After dark adaptation for 15 minutes, the leaf's initial fluorescence (F_o) in the non-photosynthetic condition was measured and then irradiated with PAR 0.1 mol m^{-2} s^{-1} (k = 650 nm) using a red light-emitting diode. The maximum fluorescence (F_m) of the leaves was then determined by applying a 0.8 s saturation pulse of PAR 9,000 mol m^{-2} s^{-1}. Variable fluorescence (F_v) and maximum photochemical efficiency (F_v/F_m) of PSII were calculated using the formula (F_m - F_o) / F_m (Sonjaroon *et al.* 2016).

2.6. Determination of Lipid Peroxidation and Hydrogen Peroxide Content

Malondialdehyde (MDA) was measured to determine the lipid peroxidation. Leaf samples were homogenized in 1 ml of 0.1% trichloroacetic acid (TCA). The supernatant was obtained by centrifugation at 10,000 xg for 20 min at 4°C, after which 0.5 ml of the supernatant was mixed with 0.5% thiobarbituric acid (TBA) in 20% TCA. The mixture was incubated at 98°C for 30 min, and the reaction was stopped by placing the mixture in an ice bath. After centrifugation at 10,000 xg for 5 min, the absorbance of the supernatant was measured at 532 and 600 nm. Thiobarbituric acid reactive substance (TBARS) was quantified using a molar extinction coefficient of 155 mmol dm^{-3} cm to calculate its concentration.

For hydrogen peroxide (H_2O_2) measurement, 0.1 gram of leaf samples were homogenized in 1 ml of 0.1% trichloroacetic acid. The homogenate was centrifuged for 15 min at 12,000 xg. For the measurement, 0.5 ml of the extracted solution was mixed with 0.5 ml of potassium phosphate buffer (10 mM, pH 7.8) and 1 ml of 1 M KI and then the supernatant mixture was measured at 390 nm.

2.7. Antioxidative Enzymatic Activity Assays

To measure the antioxidative enzymatic activity of peroxidase (POD), 0.1 gram of leaf samples were homogenized in 300 µl of 16 mM phosphate buffer (pH 7.8) containing 0.4 mM EDTA and 2% insoluble polyvinylpyrrolidone for 15 min at 4°C. The homogenized samples were centrifuged at 13,000 xg for 15 min. The supernatant was used as an enzyme extract for the POD activity assay. The protein concentration of the enzyme extract was determined according to Bradford (1976).

2.8. Statistical Analyses

All data on physiological measurements (RWC, gas exchange rate, and chlorophyll fluorescence) and biochemical measurements (MDA, H_2O_2, proline, antioxidant enzymatic activities, qPCR) were represented as mean ± standard error (n = 5). The data was analysed using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test with a probability level of 0.05 as statistically significant. qPCR experiment was repeated at five biological replicates (three technical replicates for each biological replicate). Statistical analyses were performed using IBM SPSS Statistics v. 26 software (IBM, New York, USA).

3. Results

3.1. Proline Content and Gene Expression Analysis

The accumulation of proline was measured under drought-stress conditions. Results showed that the amounts of proline continuously increased in all mulberry varieties from day 0 to day 6. Proline levels peaked on day 6 and then started to decline. Interestingly, SRCM9809-34, the drought-tolerant

variety, contained the least amount of proline throughout the 7 days of the experiment. Moreover, Srisaket 84 had the highest proline level on day 7 after a drought (Figure 1A).

In proline biosynthesis, the transcript level of the *Pyrroline-5-carboxylate reductase* (*P5CR*) gene was measured on day 5 after the drought. The expression of *P5CR* did not significantly change in the four Thai-recommended mulberry varieties, but the *P5CR* expression level significantly increased to 4.251 folds in SRCM9809-34 (Figure 1B).

3.2. Wilting Score

Wilting scores of five mulberry varieties started as zero at the beginning of the experiment. After five days without water, the Thai recommended mulberry varieties had average wilting scores of 2, 2.6, 3.4, and 3.4 in Burirum 60, Srisaket 84, Sakhonnakorn, and Sakhonnakorn 85, respectively, while the wilting score of SRCM9809-34 remained at zero. The wilting scores of the Thai-recommended mulberry varieties gradually increased on day six and day seven, reaching an average of 3.15 and 3.6,

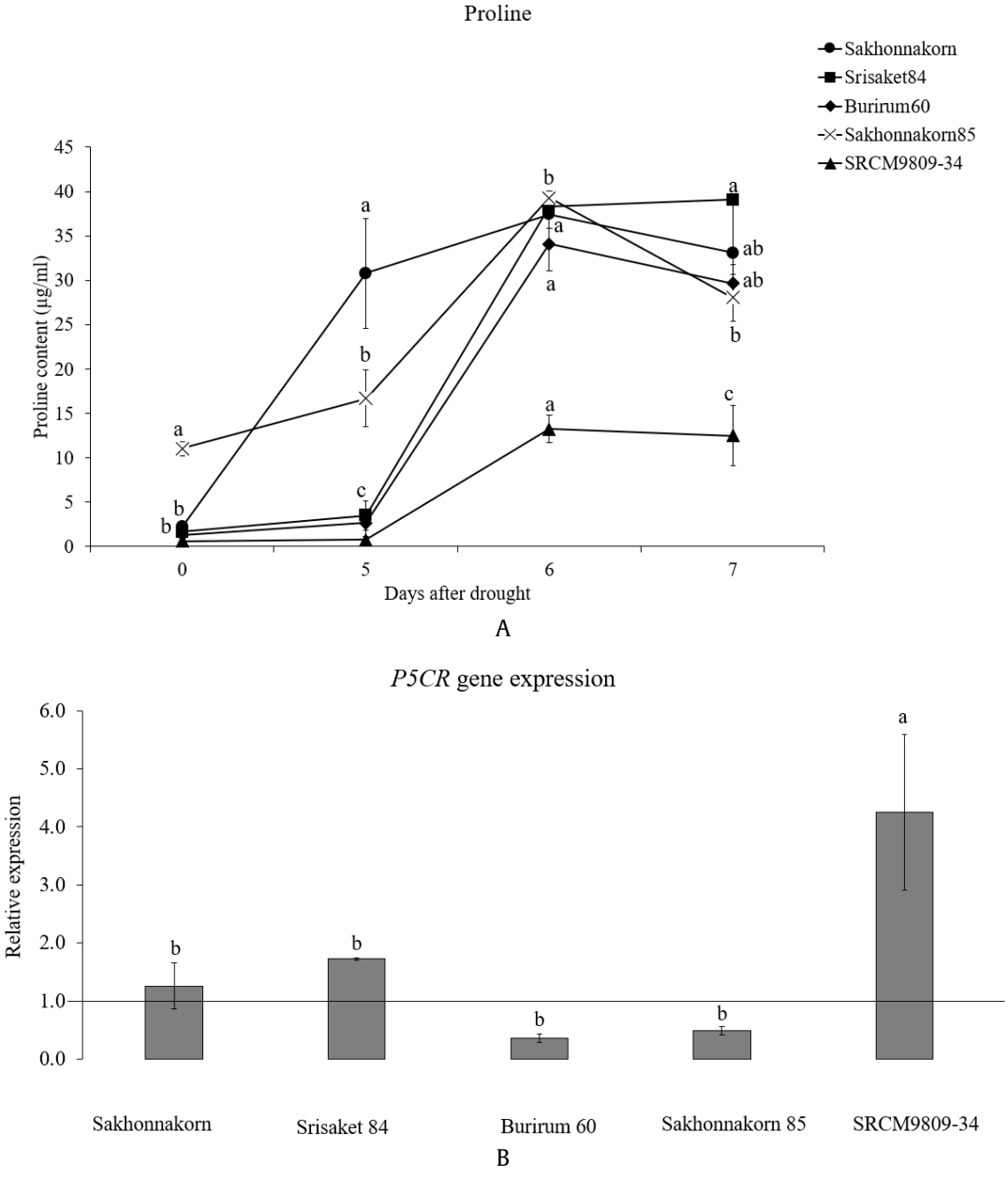

Figure 1. (A) Proline content (µg/ml) in five mulberry varieties treated for 7 days in drought conditions. Values are the means of five replicates ±S.E. Bars with the same lowercase letter are not significantly different at P<0.05 level according to Duncan's test, (B) relative expression of *P5CR* gene in five mulberry varieties on day 5 under drought conditions. Bars represent the means of biological replicates (three technical replicates per biological replicate) ±S.E. Bars with the same lowercase letter are not significantly different at P<0.05 level according to Duncan's test

respectively. On the other hand, the average wilting score of SRCM9809-34 on day seven was one (Figure 2). The phenotypes of the five mulberry varieties on day seven between control and drought treatments are displayed in Figure 3.

3.3. Relative Leaf Water Content and Chlorophyll Content

After stopping watering, the relative leaf water content (RWC) of all mulberry varieties was assessed on days 0, 5, 6, and 7. RWC gradually declined after the drought treatment. Among the five examined mulberry varieties, SRCM9809-34 displayed the highest level of RWC, while Sakhonnakorn exhibited the lowest RWC (Figure 4). The chlorophyll content was measured using a SPAD device. The findings showed that the chlorophyll content of Sakhonnakorn increased and peaked on the fifth and sixth days. The chlorophyll content of SRCM9809-34 and Sakhonnakorn 85 remained relatively constant, while the chlorophyll content of Srisaket 84 and Burirum 60 significantly reduced on the sixth and seventh days (Figure 5).

3.4. Gas Exchange and Chlorophyll Fluorescence of Mulberry Under Drought Stress

The photosynthetic rate (P_n) of all mulberry varieties showed a decline on the fifth day of the experiment. SRCM9809-34 had a higher P_n value than other varieties, while Sakhonnakorn 85 displayed the lowest daily P_n values (Figure 6).

Stomatal conductance (g_s) significantly decreased on day 5 and 7 of drought treatment. SRCM9809-34 exhibited a modest reduction in g_s values, peaking on the sixth day and decreasing immediately on the seventh. The stomatal conductance of the other varieties decreased slightly, while Burirum 60 fell on the fifth day and then increased again on the sixth and seventh day. The highest stomatal conductance of Burirum 60 was recorded on day 7, whereas a decrease was observed for the other varieties (Figure 7).

The water use efficiency (WUE) of the five mulberry varieties was not significantly different at the start of the experiment. WUE reduced rapidly in four Thai-recommended mulberry varieties on days five, six, and seven, while the WUE of SRCM9809-34 remained relatively constant (Figure 8). Moreover, on day seven, the WUE of the four Thai-recommended mulberry varieties was significantly lower than that of SRCM9809-34 (Figure 8).

All mulberry varieties had a typical range of maximum photochemical efficiency of PSII (F_v/F_m), averaging 0.81 on day 0 and 0.854 on day 5. Burirum 60 and Sakhonnakorn 85 exhibited low F_v/F_m levels at 0.75 and 0.74, respectively, on day 6. On the seventh day, all varieties exhibited F_v/F_m values less than 0.77 (Figure 9).

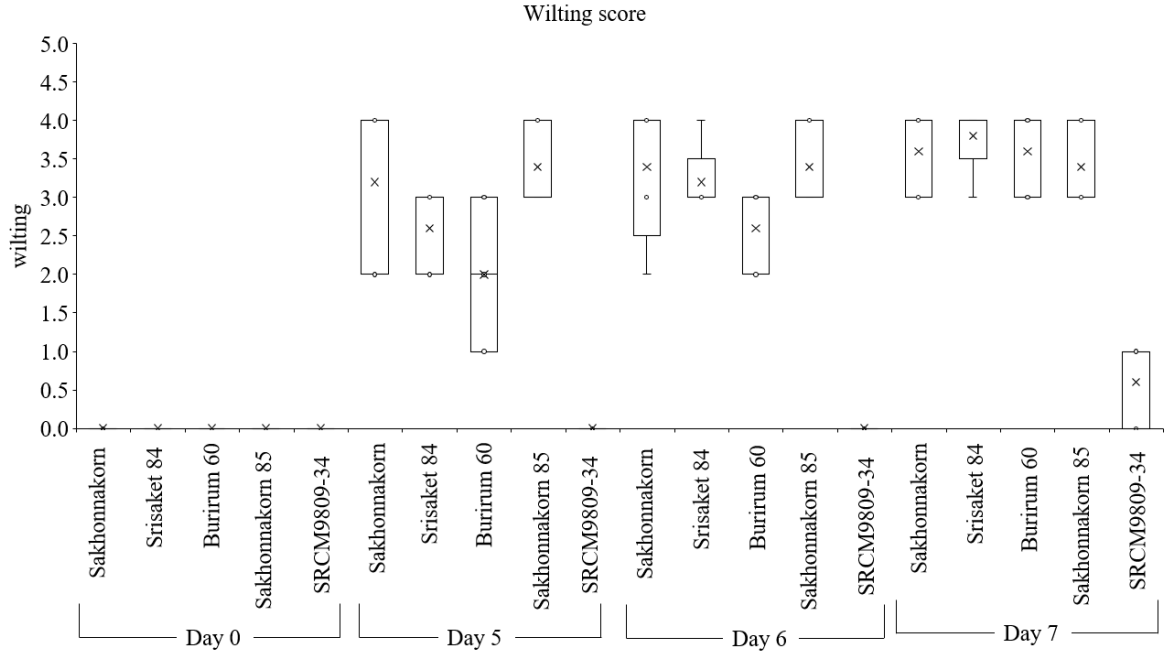

Figure 2. Boxplot of wilting score in five mulberry varieties in drought conditions. The box plots show the median, mean, first quartile, third quartile, minimum, and maximum

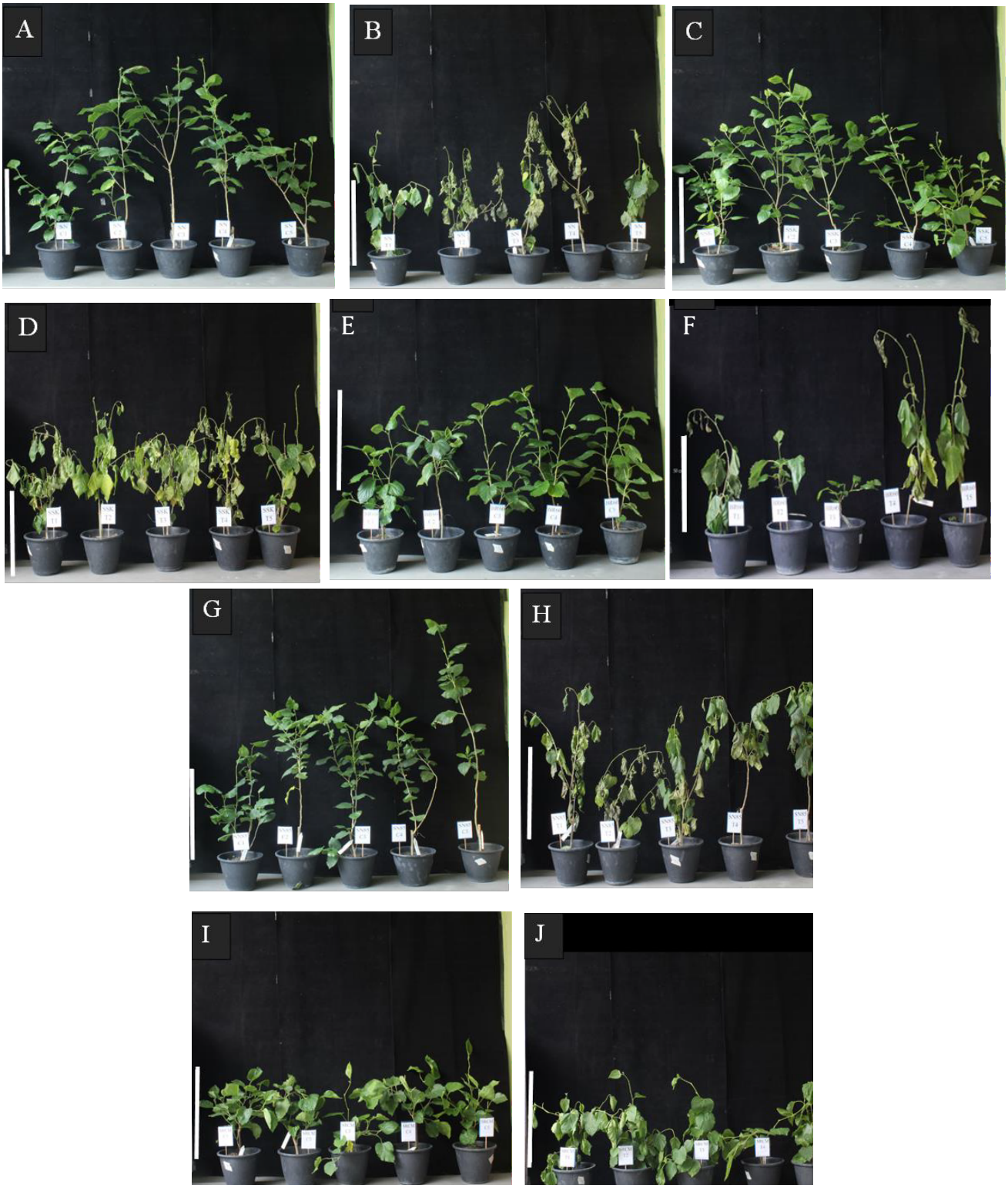

Figure 3. Mulberry plants at day 7 of drought experiment; (3A) Sakhonnakorn control, (3B) Sakhonnakorn drought, (3C) Srisaket 84 control, (3D) Srisaket 84 drought, (3E) Burirum 60 control, (3F) Burirum 60 drought, (3G) Sakhonnakorn 85 control, (3H) Sakhonnakorn 85 drought, (3I) SRCM9809-34 control, (3J) SRCM9809-34 drought. The bar = 50 cm

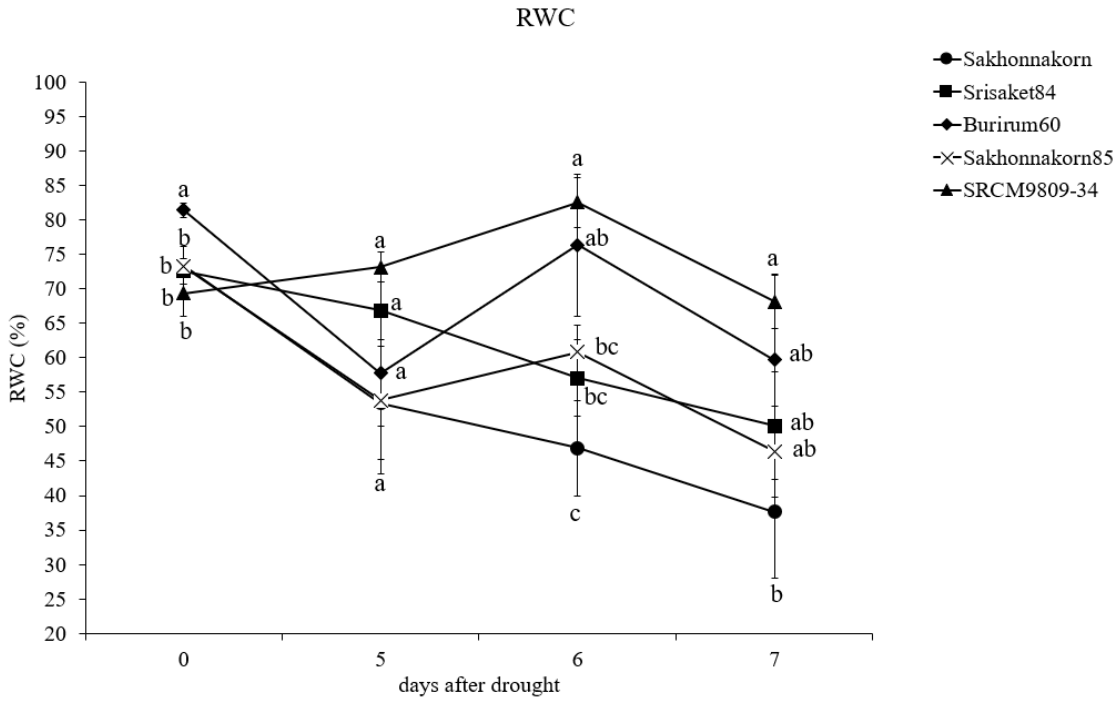

Figure 4. The percentage of relative leaf water content in five mulberry varieties treated for 7 days in drought conditions. values are the means of five replicates ±S.E. Bars with the same lowercase letter are not significantly different at P<0.05 level according to Duncan's test

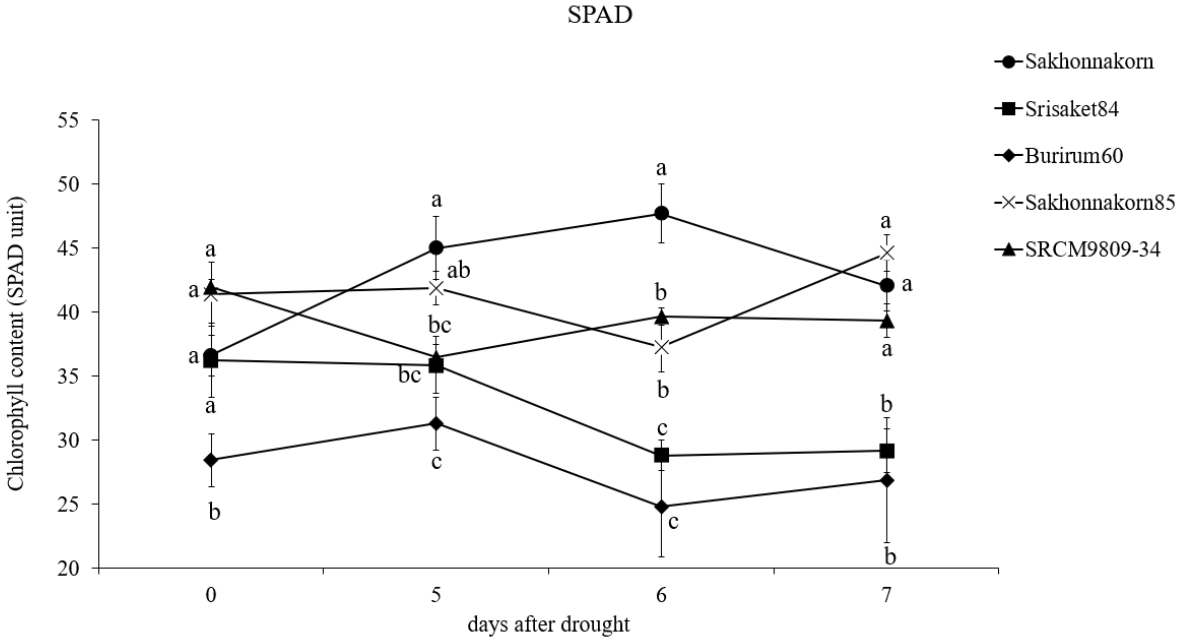

Figure 5. Chlorophyll content in the SPAD unit in five mulberry varieties treated for 7 days in drought conditions. Values are the means of five replicates ±S.E. Bars with the same lowercase letter are not significantly different at P<0.05 level according to Duncan's test

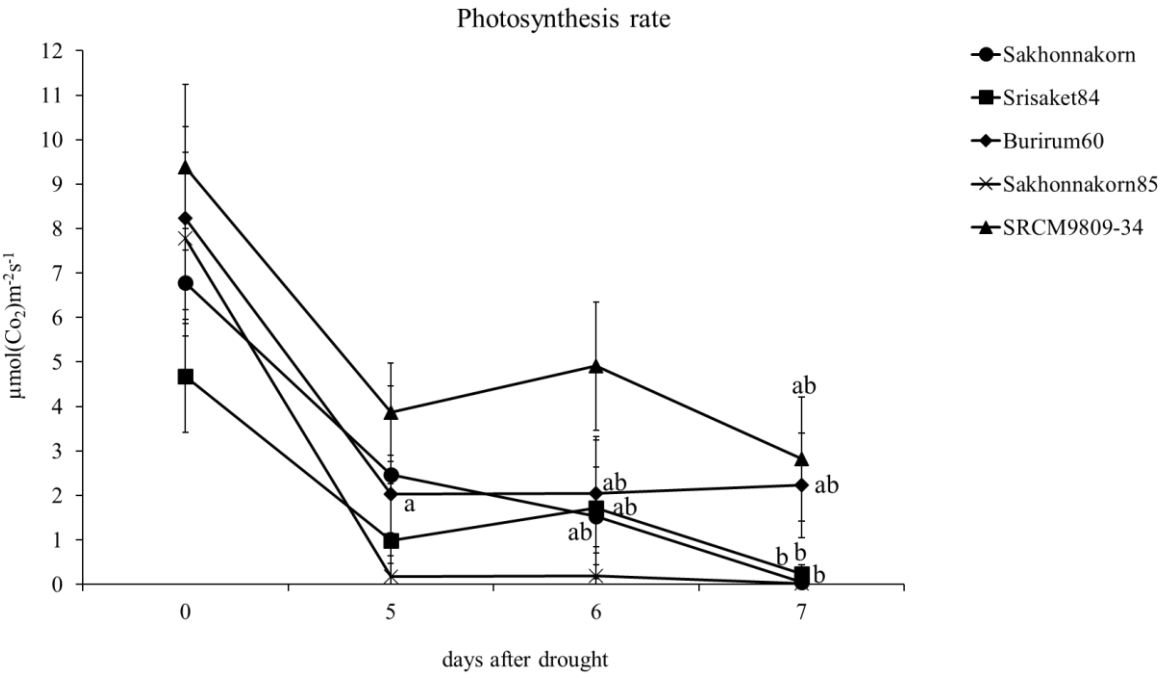

Figure 6. Photosynthesis rate (P_n) of five mulberry varieties treated for 7 days in drought conditions. Values are the means of five replicates ±S.E. Bars with the same lowercase letter are not significantly different at P<0.05 level according to Duncan's test

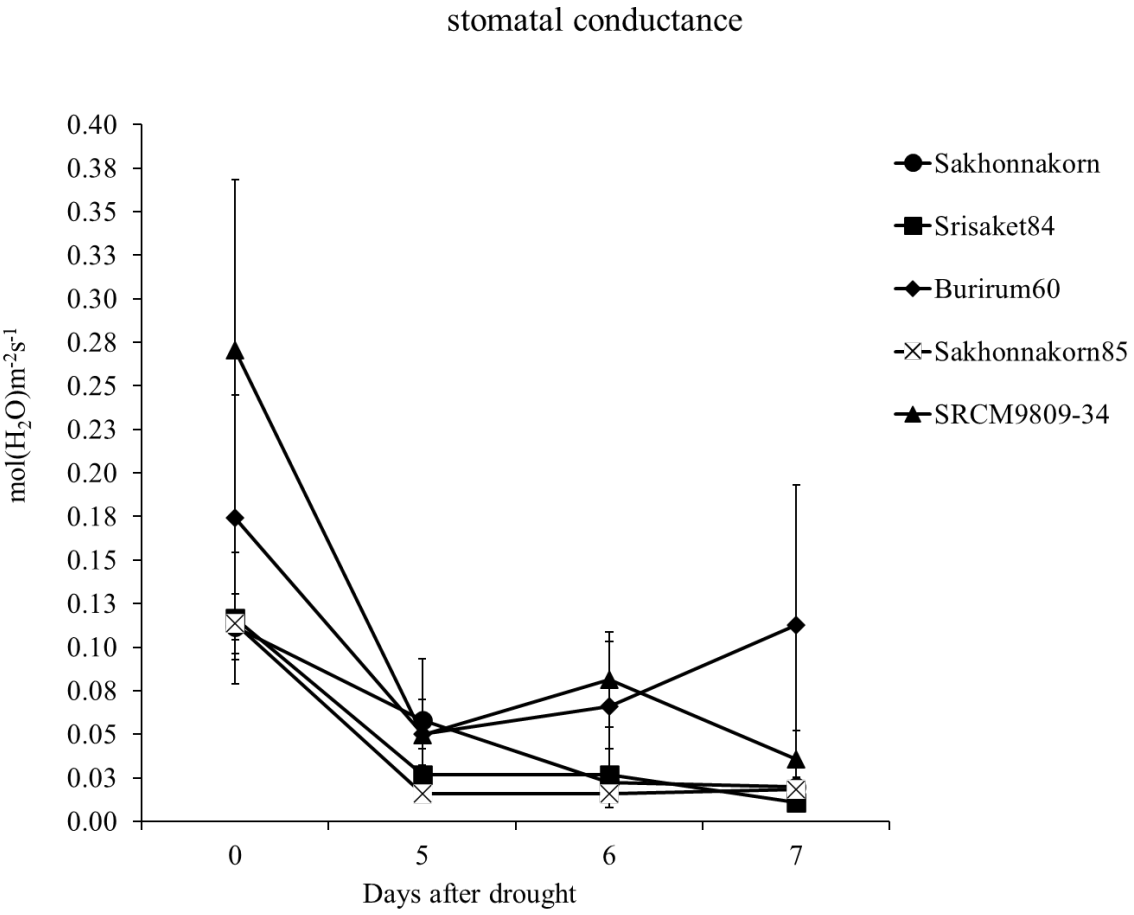

Figure 7. Stomatal conductance (g_s) of five mulberry varieties treated for 7 days in drought conditions. Values are the means of five replicates ±S.E. Bars with the same lowercase letter are not significantly different at P<0.05 level according to Duncan's test

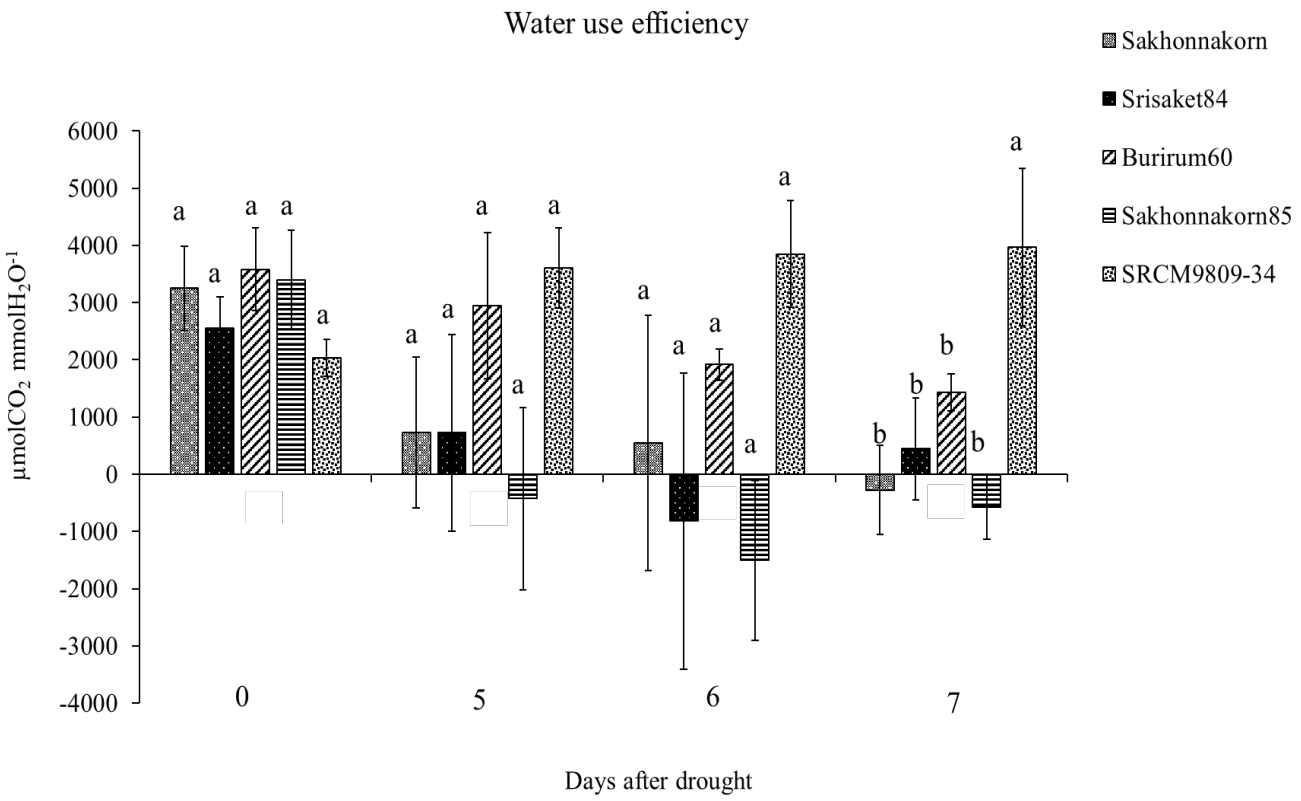

Figure 8. Water use efficiency (WUE) of five mulberry varieties treated for 7 days in drought conditions. Values are the means of five replicates ±S.E. Bars with the same lowercase letter are not significantly different at P<0.05 level according to Duncan's test

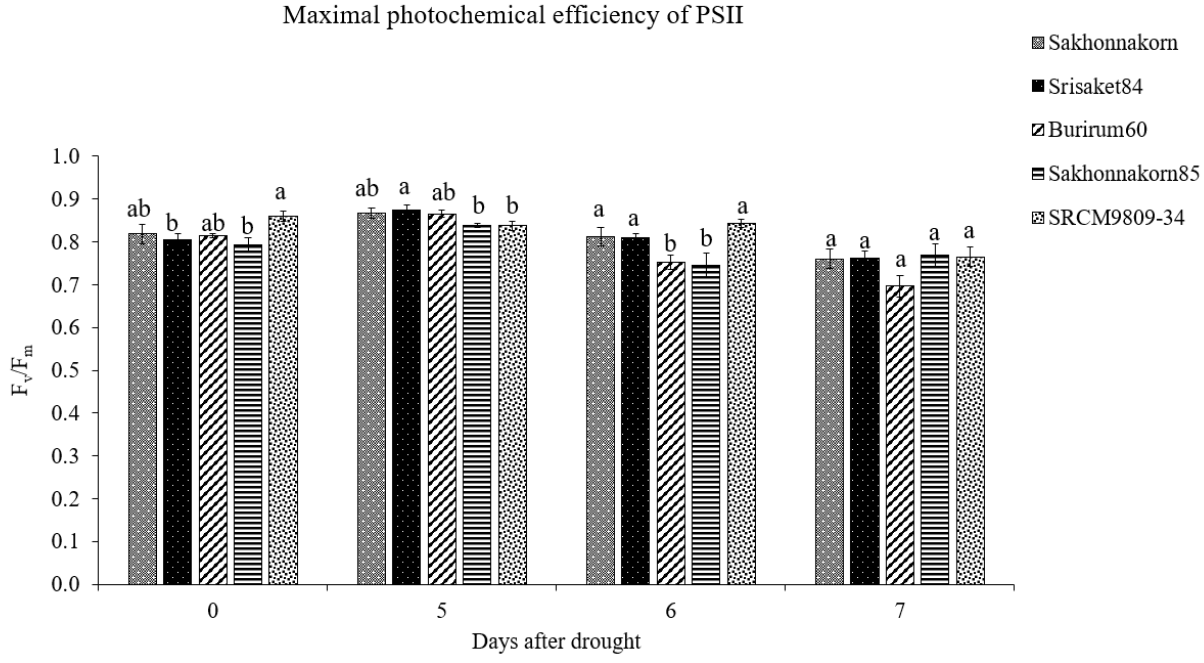

Figure 9. Maximal photochemical efficiency of PSII (F_v/F_m) of five mulberry varieties treated for 7 days in drought conditions. Values are the means of five replicates ±S.E. Bars with the same lowercase letter are not significantly different at P<0.05 level according to Duncan's test

3.5. Lipid Peroxidation, Hydrogen Peroxide Content, and Enzyme Activities of Mulberry Under Drought Stress

At the beginning of the experiment, MDA content was highest in SRCM9809-34 (27.6 nmol/g of fresh weight), followed by Sakhonnakorn 85 (16.3 nmol/g of fresh weight), Burirum 60 (13.8 nmol/g of fresh weight), Sakhonnakorn (2.8 nmol/g of fresh weight) and Srisaket 84 (1.4 nmol/g of fresh weight) respectively. After 5 days, the MDA level of all mulberry varieties dropped significantly (Figure 10A).

All mulberry varieties had a similar amount of hydrogen peroxide (H_2O_2), approximately 25 units/µg of fresh weight, at the beginning of the experiment. The H_2O_2 content gradually increased from day 0 to day 7 except for Sakhonnakorn, which showed a jump in H_2O_2 level on day 6 and came back down on day 7. SRCM9809-34 had the lowest H_2O_2 level daily (Figure 10B).

The antioxidative activity was evaluated by measuring peroxidase (POD) enzyme activity. POD activity increased after the drought treatment. SRCM9809-34 had the highest POD activity on all days examined (Figure 10C). Sakhonnakorn 85 had the lowest POD activity on day 5 and 6, but Burirum 60 had the lowest on day 7 (Figure 10C).

4. Discussion

Drought is a significant abiotic factor that can affect the growth and physiology of plants, as water is the primary constituent of living organisms. Inadequate water supply is a primary abiotic stressor that affects the overall health and growth of plants and occurs for a broad range of reasons (Singh *et al.* 2020). Proline is an essential amino acid when a plant is under stress. Proline has been shown to preserve folded protein structures from denaturation, stabilize cell membranes by interacting with phospholipids, serve as a hydroxyl radical scavenger, and provide energy and nitrogen (Claussen 2005). The phenomena are consistent with those observed in tomatoes (Claussen 2005) and wheat (Johari-Pireivatlou 2010). According to the wilting score, the initial response to drought stress is wilting because the function of turgor pressure, which expands and maintains the rigidity of plant cells, is lost. Without turgor pressure, plant leaf cells begin to collapse, resulting in a floppy appearance. Due to increased wilting, plant cells begin to collapse and ultimately die. Drought stress occurs when a plant requires more water than it can absorb at a given time (Singh *et al.* 2020). Relative leaf water content refers to the water in leaves consistent with the wilting score. Our results were consistent with previous reports that plants under drought stress consistently increased proline (Sarker *et al.* 1999). The primary function of proline is to maintain cellular water potential and provide metabolites that protect sensitive molecules under stress. As solute concentration rises, water potential falls, and water moves spontaneously from places with a high water potential to those with a low water potential. Therefore, plants with a high solute concentration will have a high water content in their cells since they have absorbed more water (Suriya-arunroj *et al.* 2004).

The amount of proline in our experiment did not correspond to the level of *P5CR* gene expression. A similar result was reported in perennial ryegrass studied by Li *et al.* (2015). Overexpression of *P5CR* does not increase proline content, suggesting that *P5CR* is not a rate-limiting enzyme in proline biosynthesis. The main gene responsible for proline synthesis exhibits variation across various plant species. The research indicates that the presence of a single gene is insufficient to serve as the sole source of the rate-limiting enzyme. Moreover, the regulation of intracellular proline levels in plants is influenced by several processes, such as production, catabolism, and intercellular and intracellular transport across different cellular compartments (Meena *et al.* 2019).

SPAD measurement of chlorophyll content showed that chlorophyll level was consistent with the level of drought tolerance, as demonstrated in previous studies including rice paddy, maize, sugarcane, and *Arabidopsis thaliana* (Bullock and Anderson 1998; Esfahani *et al.* 2008; Jangpromma *et al.* 2010; Ling *et al.* 2011). The maximum photochemical efficiency (F_v/F_m) of PSII, which assessed photosynthetic efficiency in a dark-adapted condition. For many plant species, an optimum value of F_v/F_m falls between 0.79 and 0.84, with lower values suggesting that the plant may be under stress (Choi and Jeong 2020). According to the results, if the F_v/F_m ratio is less than 0.77 on the seventh day, all varieties experience stress. Similar findings were obtained in *Erica multiflora* L. and *Pinus halepensis* L., whose F_v/F_m values varied between 0.4 and 0.8 (Prieto *et al.* 2009).

Under drought circumstances, our results demonstrated that SRCM9809-34 was a drought-tolerant variety, whereas Sakhonnakorn 85 was a susceptible variety, in agreement with Lin *et al.* (2012). Drought stress affects photosynthetic rate (P_n), stomatal conductance (g_s), and water use efficiency (WUE). Similar results were found in research on *Eucalyptus* species subjected to drought; the data indicated that g_s tended to decrease when plants were exposed to prolonged drought. The long-standing theory of stomatal behaviour predicts that species suited to dry environments with periodic droughts would have more conservative stomatal behaviour and utilize water more efficiently per unit of carbon

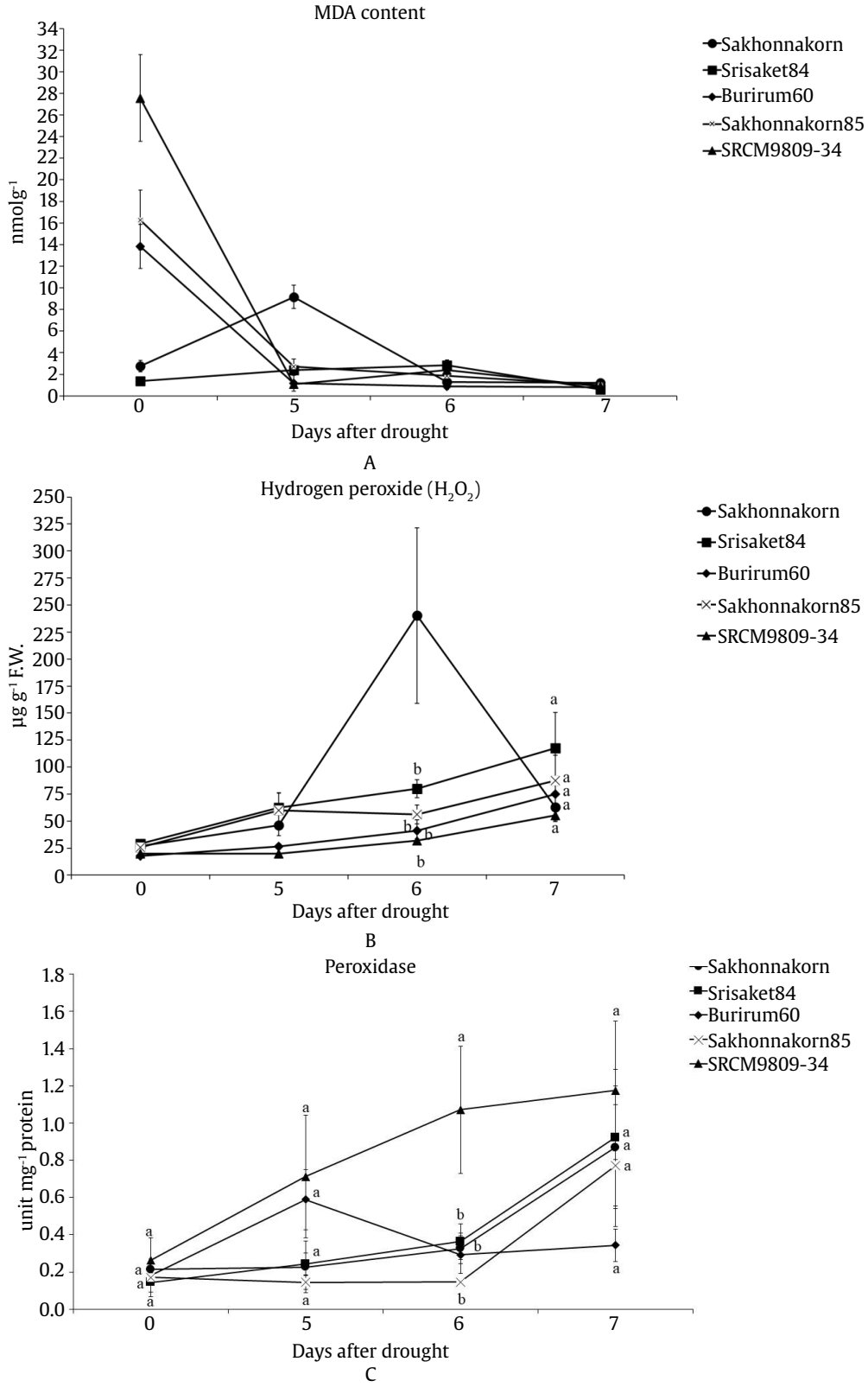

Figure 10. (A) The effect of drought stress condition on lipid peroxidation in leaves (Malonaldehyde content) of 5 mulberry varieties. Data represents the average of the experiment with three replicates. Vertical bars indicate ±S.E. Bars with the same lowercase letter are not significantly different at P<0.05 level according to Duncan's test, (B) hydrogen peroxide content (H_2O_2) of five mulberry varieties treated for 7 days in drought conditions. Values are the means of five replicates ±S.E. Bars with the same lowercase letter are not significantly different at P<0.05 level according to Duncan's test, (C) peroxidase activity (POD) of five mulberry varieties treated for 7 days in drought conditions. Values are the means of five replicates ±S.E. Bars with the same lowercase letter are not significantly different at P<0.05 level according to Duncan's test

gain than species adapted to wet habitats (Héroult *et al.* 2012). In addition, a stomatal controlled decrease in transpiration is a frequent response of plants to drought stress, and it offers a chance to improve plant water-use efficiency (Singh and Reddy 2011).

Drought stress increases the generation of reactive oxygen species (ROS), particularly H_2O_2. According to research on maize, this causes lipid peroxidation and the build-up of MDA (Mohammadkhani and Heidari 2007). MDA has been routinely used for many years as a practical biomarker for lipid peroxidation, for example, sunflower (Soleimanzadeh *et al.* 2010) and wheat (Yao *et al.* 2009). ROS may be harmful and impede plant development and metabolism. In addition, dry conditions led to an increase in the activity of peroxidase (POD), an antioxidant enzyme that minimizes ROS accumulation. In this investigation, MDA and H_2O_2 levels reached a maximum in Sakhonnakorn and sharply declined in SRCM9809-34. In contrast, POD activity reached its highest level in SRCM9809-34. Our results were similar Esfandiari *et al.* (2007) 's work on wheat seedlings.

In conclusion, SRCM9809-34 is a drought-tolerant variety; out of the four Thai mulberry varieties, Sakhonnakorn 85 showed the highest potential for drought tolerance. The findings of this study will be valuable for future in-depth analysis of mulberry drought-tolerant breeding programs.

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