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Research Article

Seed priming with hydrogen peroxide alleviates the effects of drought stress in rice (*Oryza sativa* L.) seedlings

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Abstract

Drought stress is a major factor limiting crop growth and yield. Hydrogen peroxide (H_2O_2) is known as a signalling molecule in the plant cell in which activates multiple physiological changes that play essential roles in tolerance mechanism. This study investigated the effects of seed priming with H_2O_2 on growth, some physiological characteristics and antioxidant enzyme activities in rice seedling under drought stress. Rice (*Oryza sativa* L.) cv. 'Khao Dawk Mali 105' seeds were primed with 0 (distilled water), 1, 5, 10, and 15 mM H_2O_2 and grown for 21 days. The seedlings were subjected to drought stress by withholding water for 7 days. The results showed that priming with low concentrations of H_2O_2 improved plant growth and biomass as well as relative water content, malondialdehyde content, electrolyte leakage. Priming with H_2O_2, however, had no beneficial effect on chlorophyll content, proline and leaf total soluble sugar. Seed priming with appropriate levels of H_2O_2 also enhanced antioxidant enzyme activities including superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and guaiacol peroxidase (GPX). It is concluded that seed priming with 2-10 mM H_2O_2, is beneficial for enhancing drought tolerance in rice seedling by increasing antioxidant capacity, which in turn reduces oxidative stress and damages to the cellular components.

Keywords: antioxidant enzymes; drought stress; hydrogen peroxide; rice; seed priming

Introduction

Rice (*Oryza sativa* L.) is a staple crop food for billion people worldwide, and a major export crop of Thailand. Rice production in Thailand, however, is primarily affected by drought stress because most of the rice-growing area is under rainfed condition. Drought is abiotic stress limiting growth and productivity of plants. Drought stress affects many physiological processes including growth, photosynthesis, enzyme activity, reactive oxygen species (ROS) production and membrane integrity (Farooq *et al*., 2009; Li *et al*., 2011b).

H_2O_2 is a compound generally produced as a by-product in the plant during the normal life cycle (Gill and Tuteja, 2010). However, it has been known to play a vital role in stress tolerance by acting as a secondary messenger in the plant when its level presents in low concentration (You and Chan, 2015; Farooq *et al*., 2017). The previous study suggested that H_2O_2 application in crop plants improve plant performance under stressful condition, such as *Cakile maritima,* mustard, rice variety ADT (R) 49 and Wheat (He and Gao, 2009; Mohammad and Fujita, 2013; Hameed and Iqbal, 2013; Ellouzi *et al*., 2017). H_2O_2 is the most stable molecule

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than the other ROS molecules and diffuse very rapidly across the membrane through aquaporins (Niu and Liao, 2016). Many publications reported that H_2O_2 activates mitogen-activated protein kinase (MAPK) pathway, which amplifies signalling cascade and gene modulation (Tena *et al*., 2001; Quan *et al*., 2008; Gupta *et al*., 2016; Jagodzik *et al*., 2018; Zhang *et al*., 2018). The several types of protein kinase are activated in the presence of H_2O_2 (Neill *et al*., 2002; Cruz de Carvalho, 2008; Wang and Song, 2008). In addition, H_2O_2 signalling may be involved in regulation of antioxidant enzyme activities via ABA signalling (Saxena *et al*., 2016).

Seed priming is a technique widely used for increasing germination rate, uniformity, and improving crop growth due to its simple and low-cost method (Farooq *et al*., 2009). During the priming process, internal metabolic activities are activated while the radicle protrusion is prevented. Cheng *et al*. (2017) reported that the genes related to stress including carbohydrate metabolism, protein synthesis, and signalling pathway were over-expressed during the priming stage. In addition, the advantages of seeds priming have been applied to improve the plants' performance under stress condition. According to Chen and Arora (2013) hypothesis, they proposed that primed seeds could leave "the stress memory" into the seeds. After post-priming germination, the primed seeds may perform better than the unprimed seed under stress condition. Recently, various compounds are used as priming agents such as hydropriming, halopriming (salt priming), osmopriming, thermopriming, hormones and reactive nitrogen species (Paparella *et al*., 2015; Savvides *et al*., 2016). In this study, hydrogen peroxide (H_2O_2) was used as a priming agent in order to determine its mechanism under drought stress condition.

Although the role of H_2O_2 in physiological response under stress condition has been well documented, the effectiveness of H_2O_2 in triggering stress tolerance in plants is still inconclusive. A wide range of H_2O_2 concentrations has been used and the results varied between species as well as application method. Therefore, this study aimed to investigate the potential of seed priming with H_2O_2 on some physiological characteristics of rice grown under drought stress. The effects of different concentrations of H_2O_2 were also studied in order to determine the optimum concentration of H_2O_2 used to enhance drought tolerance in rice.

Materials and Methods

Plant materials

Rice seeds cv. 'KDML 105', obtained from Khon Kaen Rice Research Center, were soaked with 1% sodium hypochlorite and rinsed with distilled water. The seeds were then primed with 0 (distilled water), 1, 2, 5, 10, and 15 mM H_2O_2 for 24 hours in dark condition at ambient temperature whereas seeds primed with distilled water were used for the control groups. The seeds were air-dried for two days after priming procedure and then sown in the pots, 8 inches in diameters and 7 inches high, filled with 3.5 kg of soil (pH 6.04, EC 0.040 ds m^{-1}, 0.0226% of total N, 36.5 mg Kg^{-1} of total P and 232.5 mg Kg^{-1} of total K). The experiment was conducted at the greenhouse at the Department of Biology, Faculty of Science, Khon Kaen University, Khon Kaen, Thailand. The experimental design was carried out by using a completely randomized design (CRD) with five replicates in each treatment. The seedlings were watered daily to the field capacity with distilled water and allowed to grow for 21 days. The seedlings were then subjected to drought stress by withholding water for 7 days in which the leaf showed the symptom of drought stress (leaf margin touch to O shape) (International Rice Research Institute, 2002). The seedlings in the control group were watered daily to the field capacity.

Sampling measurements

Plant growth and biomass

Seedlings were separated into root and shoot parts. Shoot length, root length, fresh weight (FW), and dry weight (DW) of shoot and root were recorded. For dry weight, the samples were dried in a hot air oven at

80 °C for three days and the weight of each sample was recorded. Root: shoot ratio of each sample was calculated based on dry weight.

Relative water content (RWC)

RWC was measured by the method of Turner (1981). Firstly, the fresh weight of the leaf was recorded. The leaf was put into a plate containing distilled water overnight. The fresh weight rehydrated of leaves was recorded and the leaf tissue was then dried in a hot air oven at 80 °C for three days. The RWC was calculated by using the following equation. RWC = [(FW - DW) / (rehydrated fresh weight - DW)] x 100

Electrolyte leakage (EL)

Leaf sample was soaked overnight in a test tube filled with 10 ml of distilled water. The electrolytic conductivity value was measured before boiling (EC_1). The samples were then heated on a dry bath at 100 °C for 20 minutes. After boiling, the conductivity was then measured (EC_2). The EL was calculated by using following equation EL = (EC_1 / EC_2) x 100 (Baninasab and Ghobadi, 2011).

Hydrogen peroxide content

Leaf sample (0.1 g) was grounded in liquid N_2 with 0.1% (w/v) trichloroacetic acid (TCA) and centrifuge at 4 °C 10,000 rpm for 15 minutes. Each supernatant was mix with 10 mM potassium phosphate buffer (pH 7.0) and 1 M KI. The mixtures were then placed on an ice bath in dark condition for 1 hour. The absorbance was measured by the spectrophotometer at 390 nm and the hydrogen peroxide content was calculated from a standard curve (Mishra and Agrawal, 2014).

Malondialdehyde content (MDA)

MDA content was determined according to Sunohara and Matsumoto (2004). About 0.1 g of fresh leave samples were grounded with 0.1% (w/v) TCA. The crude extract was centrifuged at 1,000 rpm for 5 minutes. The supernatant was mixed with 0.5% thiobarbituric acid (thiobarbituric acid dissolved with 20% w/v TCA). The reaction was boiled at 95 °C for 30 minutes and terminated in an ice bath for 10 minutes. The absorbance was measured at 532 and 600 nm. The concentration of TBA reacting substance (TBARS) was calculated using a molar extinction coefficient of 155 mM^{-1} cm^{-1}.

L- proline content

Proline content was measured by the method of Bates *et al*. (1973). About 0.1 g of Fresh leaf sample was grounded with 3% aqueous sulfosalicylic acid and filtrated with filter paper. Two ml of filtrate was mixed with 2.5% (w/v) acid ninhydrin (dissolved in ratio 2:3 of 6 M phosphoric acid and glacial acetic acid) and glacial acetic acid. The mixtures were boiled at 100 °C for 1 hour. The reaction was stopped by placing the test tube in an ice bath. Absorbance readings were performed at 520 nm, and the L-proline content was calculated from a standard curve.

Total chlorophyll

Chlorophyll content was determined following the method described by Arnon (1949). About 20 mg of fresh leaf sample was extracted with 5 ml of 80% acetone. The absorbance was measured at 645 and 633 nm by the spectrophotometer.

Sugar extraction and leaf soluble sugar content

About 50 mg of fresh leaf sample was extracted with 80% (v/v) ethanol. The sample was heated at 65 °C for 1 hour to collect the leaf soluble sugar. The supernatant was collected and the procedure was repeated two times.

Leaf soluble sugar content was assayed by phenol-sulfuric method described by DuBois *et al*. (1956). Sugar extract was mixed with 5% (w/v) phenol. Then, sulfuric acid was added to the mixtures and allowed to stand for 30 minutes. The absorbance was measured spectrophotometrically at 490 nm.

Activities of antioxidant enzymes

About 0.2 g of fresh leaf sample was grounded with 50 mM potassium phosphate buffer pH 7.8. The crude extract was centrifuged at 10,000 rpm for 1 min. The supernatant was then used as an enzyme extract for superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6), ascorbate peroxidase (APX; EC 1.11.1.11) and guaiacol peroxidase (GPX, EC 1.11.1.7) assays (Lu et al. 2009). The amount of protein in the enzyme extract was determined by the Bradford method (Bradford, 1976).

Catalase (CAT) activity was assayed by measuring the rate of disappearance of H_2O_2 using the method described by (Chandlee and Scandalios, 1984). The reaction mixture contained 50 mM potassium phosphate buffer pH 7.0, 0.5 M H_2O_2 and of enzyme extract. The subsequence decomposition of H_2O_2 was observed at 240 nm (E = 0.0394 mM^{-1} cm^{-1}).

Superoxide dismutase (SOD) activity was assayed by measuring its ability to inhibit the photochemical reduction of nitro blue tetrazolium chloride using the method of (Beauchamp and Fridovich, 1971). The reaction mixture (2 ml) contained 50 mM potassium phosphate buffer (pH 7.8), 10 mM methionine, 50 μM NBT, 0.025% Triton X-100 and 10 μM riboflavin. The photo-reduction of NBT was measured at 560 nM. One unit of SOD was defined as the amount of enzyme that produced 50% inhibition of NBT.

Guaiacol peroxidase (GPX) and ascorbate peroxidase (APX) activities were assayed by the method of (Nakano and Asada, 1981). For GPX assay, the reaction mixture contained 50 mM potassium phosphate buffer pH 7.8, 0.5 M H_2O_2, 3% guaiacol and enzyme extract. GPX activity was calculated from the formation of tetraguaiacol per minute at 470 nm (E = 26.6 mM^{-1} cm^{-1}). For APX assay, the reaction mixture contained 50 mM potassium phosphate buffer pH 7.0, 0.5 M H_2O_2, 20 mM ascorbic acid, 16 mM EDTA and enzyme extract. The subsequent decrease in ascorbic acid was observed at 290 nm (E = 2.8 mM^{-1} cm^{-1}).

Statistical analysis

Data was analysed using SPSS version 19.0. The data from each treatment were subjected to analysis of variance (ANOVA) and the mean difference with Duncan's new multiple range test with significant level at $p < 0.05$.

Results

Plant growth and biomass

Drought stress significantly reduced rice seedling growth as indicated by the reduction of root length, shoot length, root and shoot fresh weights, and root and shoot dry weights. Root:shoot ratio was slightly higher when the seedling was subjected to drought stress. Priming with 1, 2, 5, and 10 mM H_2O_2 showed slight increases in root and shoot length compared to unprimed seedling, while priming with 15 mM H_2O_2 caused reduction in root and shoot length. The seedlings also had higher root and shoot fresh weights when primed with 1, 2 and 5 mM H_2O_2 compared to unprimed group but were lower in 10 and 15 mM H_2O_2. Similarly, root and shoot dry weights were also higher in the seedlings primed with 1, 2 and 5 mM H_2O_2 but were lower in the seedlings primed with 10 and 15 mM H_2O_2. Root:shoot ratio in the seedlings primed with all concentrations of H_2O_2 was not significantly different from the unprimed seedling, but were slightly lower in the seedlings primed with 10 and 15 mM H_2O_2 (Table 1).

Table 1. Root and shoot length, dry weight and Root: shoot ratio of rice seedlings grown after exposed to drought stress. (n = 5)

Treatments	Seedling length (cm)		Fresh weight (g)		Dry weight (g)		Root:Shoot ratio
	Root	Shoot	Root	Shoot	Root	Shoot	
Control	19.91 ± 0.84 a	72.91 ± 0.53 b	1.78 ± 0.16 b	4.13 ± 0.18 b	0.47 ± 0.05 c	0.84 ± 0.04 c	0.55 ± 0.04 a
0 mM	19.06 ± 0.78 a	69.00 ± 1.76 ab	0.65 ± 0.07 a	1.35 ± 0.22 a	0.36 ± 0.03 ab	0.58 ± 0.03 ab	0.62 ± 0.03 a
1 mM	20.29 ± 0.71 a	71.75 ± 1.23 b	0.73 ± 0.04 a	1.51 ± 0.16 a	0.41 ± 0.02 bc	0.66 ± 0.02 ab	0.62 ± 0.04 a
2 mM	19.90 ± 1.05 a	72.82 ± 1.07 b	0.75 ± 0.06 a	1.88 ± 0.27 a	0.38 ± 0.03 abc	0.65 ± 0.03 ab	0.60 ± 0.05 a
5 mM	20.46 ± 0.79 a	72.58 ± 1.21 b	0.75 ± 0.06 a	1.52 ± 0.21 a	0.37 ± 0.02 ab	0.64 ± 0.03 ab	0.59 ± 0.03 a
10 mM	19.72 ± 1.03 a	70.06 ± 1.60 ab	0.54 ± 0.07 a	1.25 ± 0.31 a	0.32 ± 0.04 ab	0.59 ± 0.08 ab	0.55 ± 0.02 a
15 mM	18.99 ± 0.82 a	66.88 ± 1.30 a	0.56 ± 0.04 a	1.23 ± 0.14 a	0.31 ± 0.02 a	0.54 ± 0.02 a	0.56 ± 0.03 a

Note: Means with different letters within the same column indicate a significant difference according to Duncan's multiple range test ($P < 0.05$).

Some physiological characteristics

Drought stress dramatically increased electrolyte leakage compared to the control plant. Priming with 1 mM H_2O_2 slightly decreased electrolyte leakage in the leaf and priming with 2, 5, 10, and 15 mM H_2O_2 significantly lowered electrolyte leakage compared to unprimed seedling (Figure 1A). Relative water content was significantly lower when the seedling was subjected to drought stress. Priming with all concentrations of H_2O_2 improved relative water content in the leaf in which priming with 1, 5, and 10 mM H_2O_2 resulted in a significantly higher relative water content compared to unprimed seedling (Figure 1B). Total chlorophyll content was greatly reduced after the seedling was subjected to drought stress compared to the control plant. Nevertheless, total chlorophyll contents in the seedling primed with all concentrations of H_2O_2 were not significantly different from the unprimed and were significantly lower compared to the control plant (Figure 1C).

H_2O_2 content in the leaf was slightly higher in the droughted plant compared to the control group. Although priming with all concentrations of H_2O_2 resulted in lower H_2O_2 content, their levels are not significantly different compared to unprimed seedling (Figure 1D). Drought stress also increased MDA content in the leaf compared to the well-watered plant. Priming with all concentrations of H_2O_2 showed significantly lower MDA content compared to the unprimed treatment (Figure 1E).

Leaf proline content was dramatically increased when the plant was subjected to drought stress. Priming with H_2O_2 resulted in lower proline content compared to unprimed seedling in which the lowest proline content was observed when priming with 10 mM H_2O_2 (Figure 1F). Leaf total soluble sugar was also greatly reduced in unprimed group. Priming with H_2O_2 showed a slight increase in leaf total soluble sugar but their levels were not significantly different (Figure 1G).

Antioxidant enzyme activities

SOD activity was reduced by drought stress. Priming with H_2O_2 slightly increase SOD activities in all concentrations in which 10 mM resulted in the highest activity (Figure 2A). There was no significant difference in CAT activity between control and unprimed treatment. Priming with 1 mM and 10 mM H_2O_2 showed increases in CAT activities compared to the unprimed group whereas other concentrations showed no difference in CAT activities (Figure 2B). Priming with H_2O_2 also increased APX activity in all treatments. The seedling primed with 2, 5, 10 and 15 mM H_2O_2 showed significantly higher APX activities compared to

unprimed seedling (Figure 2C). Similarly, while drought stress slightly increased GPX activity of unprimed seedling, priming with all concentrations of H_2O_2 also increase GPX activity in which priming with 2, 10 and 15 mM H_2O_2 resulted in significantly higher GPX activities compared to the unprimed treatment (Figure 2D).

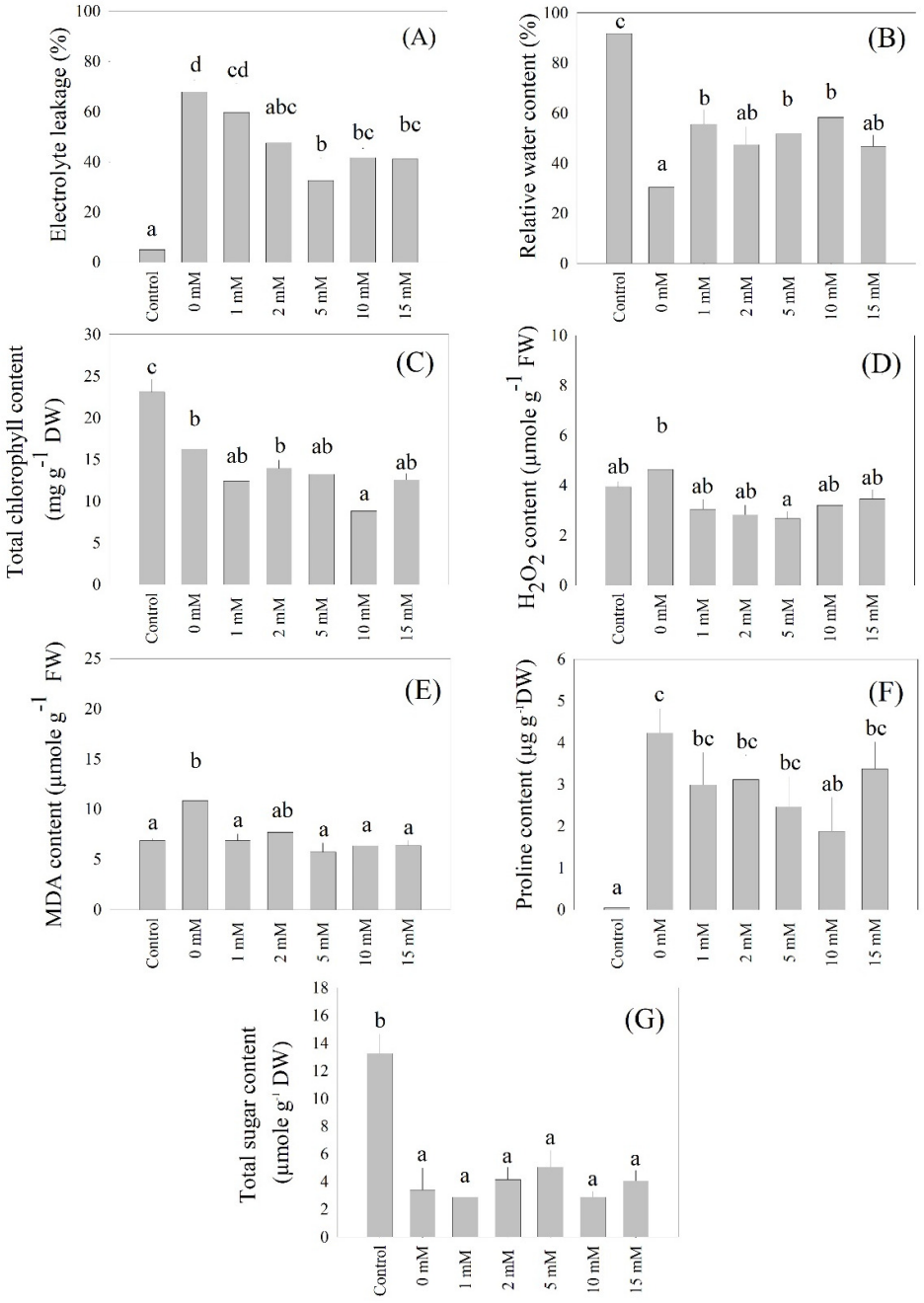

Figure 1. The effects of various concentration of H_2O_2 priming on physiological traits including (A) electrolyte leakage (B) relative water content (C) total chlorophyll content (D) H_2O_2 content (E) MDA content (F) proline content and (G) total sugar content of rice seedlings cv. 'KDML 105' under drought stress. Data are shown as the mean (± SE) of five replications

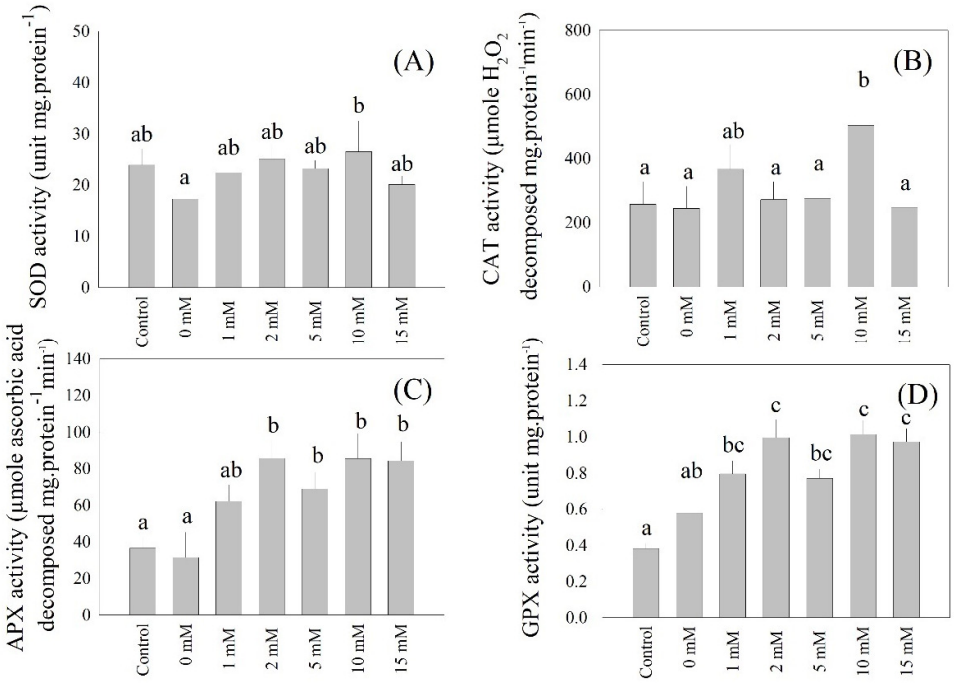

Figure 2. The effects of with various concentration H_2O_2 priming on antioxidant enzymes activities including (A) SOD (B) CAT (C) APX and (D) GPX of rice seedlings cv. 'KDML 105' under drought stress. Data are shown as the mean ± SE of five replications. Means with different letters indicate a significant difference according to Duncan's multiple range test ($P < 0.05$)

Discussion

Drought stress fundamentally impacts plant growth and affects plant productivity by impairing many physiological processes, including photosynthesis, cell expansion, nutrient uptake, as well as affecting gene expression. H_2O_2 and associated ROS are generally occurred during stress which leads to oxidative stress, thus results in cellular damage and dysfunction. Despite their toxicity, H_2O_2, in particular, is often linked with the subsequent induction of defence mechanisms which account for stress tolerance. The role of H_2O_2 in signalling during acclimation to stress has received much attention during the past decade and it has been shown that pre-treatment with low concentrations of H_2O_2 can induce protection against subsequence severe oxidative or abiotic stress, a process known as priming (Smirnoff and Arnaud, 2019; Kerchev *et al*., 2020). Many studies reported that seed priming with H_2O_2 increases stress tolerance in several plants such as cucumber, wheat and maize (Li *et al*., 2011a; Terzi *et al*., 2014; Sun *et al*., 2016). In this study, we reported that seed priming with 1-5 mM H_2O_2 mitigates the effect of drought stress in terms of plant growth while priming with high concentrations of H_2O_2 has no positive effect, but further reduces plant growth. The effects of H_2O_2 in plant stress responses are dose-specific and it has been widely accepted that low concentrations of H_2O_2 may serve as a signalling molecule (Černý *et al*., 2018). It is speculated that H_2O_2 induces molecular changes during the priming period which, in turn, triggers mechanisms that help the plant acclimate to drought stress during the seedling stage.

Seed priming with optimum concentrations of H_2O_2 also alleviates the effects of drought stress by improving various physiological characteristics. The plant treated with H_2O_2 showed higher RWC compared to the untreated plant under the drought condition. The previous study in cannabis found that H_2O_2 induced better RWC in a well-watered condition (Golizadeh *et al*., 2015). H_2O_2 priming also benefits to RWC of

Caklie martima in both drought and salt stress conditions (Ellouzi *et al*., 2017). Besides, overproduction of ROS under drought stress leads to oxidative damage to macromolecules and cellular structures which, in turn, inhibit plant growth and development. In this study, priming with H_2O_2 leads to lowered electrolyte leakage, lowered MDA content, and lowered internal H_2O_2 content in the leaf. Similarly, previous studies have shown that priming with H_2O_2 decreased MDA content as well as lowered the production of internal H_2O_2 compared to control (Hameed and Iqbal, 2013; Mohammad and Fujita, 2013). It is plausible to assume that H_2O_2 priming increased the antioxidant capacity of the treated plant, and alleviates some of the oxidative stress and membrane damage under drought stress (Hossain *et al*., 2015).

Another important aspect of drought tolerant mechanism is an osmotic adjustment, the mechanism in which the plants usually adjust water potential in the cells by accumulating compatible solutes in response to the low amount of soil water content. In this study, the seedling accumulates a large amount of proline in response to drought, while leaf soluble sugars are reduced. Proline accumulation benefits to increase water potential as well as stabilizing macromolecules such as proteins and enzymes (Kaur and Asthir, 2017). Mohammadkhani and Heidari (2008) suggested that each compatible solute is accumulated in different parts; for example, proline tends to accumulate primarily in the leaf part, but sugar is in the root part. Nevertheless, our results showed that seed priming with H_2O_2 does not enhance proline or sugar accumulation in the drought condition. It may be due to either H_2O_2 has no direct effect on the osmotic adjustment mechanism, or the plant may experience less stress in which supported by an increase in RWC and lowered MDA and electrolyte leakage.

Priming with H_2O_2 also involves in the accumulation of tolerance signalling networks such as transcription factors, activation of antioxidant capacity, and ROS scavenging (Paparella *et al*., 2015; Savvides *et al*., 2016). During the priming period, accumulation of these signalling may be imprinted in the seeds and enhance stress tolerance after post-priming germination. Our results showed that seed priming with H_2O_2 increases antioxidant enzyme activities, including SOD, CAT, APX, and GPX. Ellouzi et al. (2017) also reported that H_2O_2 priming in *Caklie martima* significantly induced activity of SOD, GPX and CAT compared to the untreated plant. It is, however, suggested that the responses of H_2O_2 treatment may depend on plant species. Ellouzi *et al*. (2017) found that H_2O_2 priming in *Caklie martima* significantly induced activity of SOD, GPX and CAT compared to control; in contrast, antioxidant enzyme activity of *Eutrema salsugineum* was slightly changed although treated in the same condition. Nevertheless, it is suggested that seed priming with H_2O_2 could trigger the activation of defence mechanism, such as increased levels of antioxidants, during priming period which persists in the seedling and help to improve the ROS scavenging capacity.

Conclusions

In conclusion, it is seen that seed priming with appropriate levels of H_2O_2, i.e., 2-10 mM H_2O_2, is beneficial for enhancing drought tolerance in rice seedling. During the priming period, exogenous H_2O_2 may act as a signalling molecule that triggers multiple defence mechanisms, such as ROS signalling, antioxidant activation, and osmotic adjustment. This “priming memory” persists in the seedling and induces various metabolic changes such as ROS signalling, antioxidant activation, and osmotic adjustment. Seed priming with H_2O_2 increases antioxidant enzyme activities, which results in lower oxidative stress and membrane damage, thus improving plant water status as well as plant growth under drought stress.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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