Effects of Seed Priming on Proline Accumulation and Antioxidant Enzymes in Rice cv. KDML 105 (*Oryza sativa* L.) Under Drought Stress

ผลของการแช่เมล็ดต่อการสะสมโพรลีน และกิจกรรมของเอนไซม์ต้านอนุมูลอิสระ ในข้าวขาวดอกมะลิ 105 ภายใต้สภาวะแล้ง

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ABSTRACT

Drought stress usually affects northeast region of Thailand resulting in lower rice yield. This research aimed to study the effects of seed priming in different agents including distilled water (hydropriming), 10% polyethylene glycol, 10 µl spermine, 2 mM ascorbic acid, 100 mM glycine betaine and 10 mM $CaCl_2$ in rice seedling cv. Khao Dawk Mali 105 under drought stress. Rice seeds were primed with the chemicals for 48 hours in ambient temperature. Rice seedlings were grown hydroponically in half-strength Hoagland's solution pH 5.0 for 21 days. Seedlings were then subjected to drought stress by adding 15% polyethyleneglycol to the nutrient solution, except the control group which was grown in the typical nutrient solution. Under drought stress, the results showed that seed priming agents improved relative water content, proline accumulation and activity of antioxidant enzymes compared to hydropriming. Therefore, it can be concluded that seed priming with the chemicals can enhance drought tolerance in plants.

บทคัดย่อ

ภาคตะวันออกเฉียงเหนือเป็นพื้นที่หนึ่งที่ประสบปัญหาจากสภาวะเครียดแล้งซึ่งส่งผลต่อปริมาณผลผลิตข้าว โดยในการศึกษาครั้งนี้มีวัตถุประสงค์เพื่อเปรียบเทียบผลของสารเคมีต่าง ๆ ได้แก่ น้ำกลั่น 10% โพลีเอททิลีนไกลคอล 10 µl สเปอร์มีน 2 mM กรดแอสคอร์บิก 100 mM ไกลซีนบีเทน และ 10 mM แคลเซียมคลอไรด์ ในข้าวขาวดอกมะลิ 105 โดยทำการแช่เมล็ดเป็นเวลา 48 ชั่วโมงที่อุณหภูมิห้อง จากนั้นย้ายเมล็ดข้าวที่ทำการแช่เมล็ดแล้วลงปลูกในสารละลายธาตุอาหาร Hoagland เข้มข้นครึ่งหนึ่ง pH 5.0 เป็นเวลา 21 วัน จากนั้นจึงชักนำให้ต้นกล้าข้าวอยู่ในสภาวะแล้งโดยการเติมโพลีเอททิลีนไกลคอล 15% ในสารละลายธาตุอาหาร ในขณะที่กลุ่มควบคุมที่ได้รับน้ำตามปกติจะปลูกในสารละลายธาตุอาหารปกติ ซึ่งผลการทดลองพบว่าภายใต้สภาวะแล้ง เมล็ดที่ได้รับการแช่ด้วยสารเคมีจะมีค่าปริมาณน้ำสัมพัทธ์ในใบ ปริมาณโพรลีน และกิจกรรมของเอนไซม์ต้านอนุมูลอิสระสูงขึ้นเมื่อเปรียบเทียบกับเมล็ดที่แช่น้ำกลั่นเพียงอย่างเดียว ดังนั้นการแช่เมล็ดจึงเป็นอีกวิธีหนึ่งที่ช่วยในการทำให้พืชทนต่อสถาวะแล้งได้ดีขึ้น

Keywords: Seed priming, drought stress, antioxidant enzymes

คำสำคัญ: การแช่เมล็ด ความเครียดแล้ง เอนไซม์ต้านอนุมูลอิสระ

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Introduction

Rice (*Oryza sativa* L.) is an important crop that widely consumed as a staple food for about half of human's population. Rice cultivar Khao Dawk Mali 105 (KDML105) is abundantly cultivated in rainfed area of northeast and northern of Thailand as well as areas across central, eastern and parts of eastern of Thailand (Bereau of rice research and development, n.d.). Morover, it is abundantly cultivated due to it fragrance and high cooking quality (Bereau of rice research and development, 2016). In 2016, 9,883,289 tons of rice was exported which worth 154,434 million Baht (Thai rice exporters association, 2016a). In Thailand, total area under rice is estimated about 56.3 million Rai, Mali rice representing approximately 28.33 million Rai of rice growing area (Rice department, 2016). In recent years, rice production was declined due to irrigate restriction, low precipitation and drought (United States Department of Agriculture, 2015). In addition, climate change is now recognized as one of major threats for the planet earth in 21th century (Mishra, Singh, 2010). Rice growing depends on irrigation supplies and precipitation in which this phenomenon may affect rice production. Historical export statistic showed that rice yield decreased from 31.36 million tons to 27.42 million tons in 2016 (Thai rice exporters association, 2017b).

Under drought stress, plants growth is largely inhibited due to loss of turgor pressure. In addition, plants' metabolism is changed under drought condition due to overproduction of reactive oxygen species (ROS) which interacts with lipids, DNA and proteins. As a result, plant cells are damaged and the normal function is impaired.

Various methods were reported to increase drought tolerance. Seed priming is recommended as one method due to its simplicity and low cost. Recently, seed priming was applied to increase the tolerance of plant by using various conditions e.g. osmopriming, halopriming, thermopriming and oxygen-nitrogen-sulfur species priming (Dalil, 2014; Savvides et al., 2016). Seed priming is a technique that seeds were partially hydrated to a point which begins metabolic processes but the emergence of radical does not occur (Farooq et al., 2009). Primed seeds were shown better uniformity and germination rate when compared to non-primed seeds by leading molecular changes in seeds (Hussian et al., 2014). Seed priming involved with 3 phases; including (I) imbibtion phase, (II) lag phase and (III) germination phase. Imbibition phase includes rapid water uptake, many cellular processes are activated such as DNA repairing and ATP production (Hussian et al., 2014; Paparella et al., 2015). Lag phase involved a net little water uptake (Eskandari, 2013) and known as critical process for seed priming, various metabolic processes are activated along with new physiological activities involved with germination. For example in rice priming, there have been reported that expression of genes related to stress, carbohydrate metabolism, protein synthesis and signaling were increased during priming (Cheng et al., 2017). Germination phase is indicated by an increase of water uptake coupled with radicle protrusion and growth. In addition, seed priming was reported to improve drought tolerance by increasing content of compatible solutes and antioxidant enzymes during priming. Moreover, primed seeds which were triggered by moderate stress during priming also shown better performance under stress condition. As a results, primed seeds may imprint stress memory mediated by proteins, transcription factors and epigenetics changes and may leave in seed (Chen, Aurora, 2013; Hussian et al., 2014).

Physiological mechanism including osmotic adjustment and antioxidant system have been the most important mechanisms responsible for drought tolerance. Under drought stress, plants always accumulate the

compatible solutes which include proline, soluble sugars, glycine betaine or proteins to maintain the water potential in plant cell. In addition, high activity of antioxidant enzymatic system constituents to scavenging ROS to alleviated oxidative stress (Farooq et al., 2009). Thus, high level of proline accumulation and antioxidant enzymes activities may be used as drought tolerant indicator.

Many researchers have been reported on using many kinds of agents in seed priming including plant growth regulators, salts and other agents. A few studies have compared the potential of those agents on proline accumulation and the activity of antioxidant enzymes. In this study, six different agents including distilled water (hydropriming) polyethyleneglycol (PEG), spermine (SPM), ascorbic acid (ASA), glycine betaine (GB) and $CaCl_2$ (CC) were investigated. Previous studies have proven that these agents contribute to drought tolerance of plants. Seed priming with PEG increased early seedling growth in barley as well as improving root length and activity of superoxide dismutase in rice under drought stress (Li, Zhang, 2012; Amini, 2013). Roles of SPM, ASA and GB are involved in antioxidant system, maintain membrane integrity and cellular structure (Ashraf, Foolad, 2006; Farooq et al., 2013; Pál et al., 2015). Furthermore, GB was also reported that it plays a vital role as an osmoprotectant in plant cell (Ashraf, Foolad, 2006). It is interesting that Ca salts, especially $CaCl_2$, can lead to better crop yield and development of cereals under stressful conditions (Bismillah Khan et al., 2015). Thus, different agents including distilled water, PEG 6000, SPM, ASA, GB and CC were investigated to study the potential of seed priming on relative water content, proline content and the activity of antioxidant enzyme of rice cv. KDML105 under drought stress.

Objectives of the study

The propose of this research was aimed to studied the effects of seed priming, including distilled water, PEG 6000, SPM, ASA, GB and CC, on relative water content, proline accumulation and the activities of antioxidant enzymes in rice cv. KDML 105 seedlings under drought stress.

Methodology

Plant materials

Rice seeds (*Oryza sativa* L.) cv. Khao Dawk Mali 105 (KDML 105) were used in this study. Seeds were surfaced-sterilized with 1% sodium hypochlorite for 3 minutes and rinsed with distilled water 3 times. Distilled water (hydropriming), 10% PEG, 10 µM SPM, 2 mM ASA, 100 mM GB and 10 mM CC were applied for seed priming. Rice seeds were primed for 48 hours in ambient temperature. After pre-soaking treatment, seeds were rinsed with distilled water 3 times. The seeds were sown for 5 days to obtain seedlings. The seedlings were transferred to half-strength Hoagland's solution. The pH of nutrient solution was adjusted to 5.0. Twenty-one-days-old seedlings were subjected to 15% PEG solution for drought stress. Meanwhile hydroprimed seedlings were grown in the typical nutrient solution without adding 15% PEG as the control group. Seedlings were harvested after subjected to drought stress for 10 days. Each treatment was 5 replicates.

Measurement of relative water content (RWC)

Relative water content was measured by method of Turner (1981). Fresh leaf sample were cut into size of 2-3 cm. Fresh weight of leaves was recorded. The leaves were put into plate which containing 10 ml of distilled water and then the plates were kept at 4 ˚C overnight. The saturated fresh weight leaves were recorded and then the leaf tissues were dried in hot air oven at 80 ºC for 3 days. The RWC was calculated by using following equation.

RWC = [(fresh weight – dry weight)/(saturated fresh weight – dry weight)] x 100

The unit expressed as percent of RWC.

Measurement of proline content

Proline content was measured by method of Bates et al. (1973). Fresh leaf samples of 0.1 g were grounded with 5 ml of 3% aqueous sulfosalicylic acid and then the crude extraction was filtrated with No.2 Whatman filter paper. Two ml of filtrate was mixed with 2 ml of acid ninhydrin and 2 ml of glacial acetic acid then the test tubes were covered with parafilm. The test tubes were boiled in 100 ºC for 1 hour. The reaction was terminated in ice bath. The reaction mixture was extracted with 4 ml of toluene then mixed vigorously. Two ml of toluene phase was measured at 520 nm.

Protein and antioxidant enzymes extraction

In this study, four of antioxidant enzymes were measured including superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and guaiacol peroxidase (GPX) respectively. Fresh leaf samples (0.2 g) were grounded with 4 ml of grinding buffer containing 50 mM potassium phosphate buffer pH 7.8, 0.4 mM EDTA and 1 mM ascorbic acid, in ice bath. The suspension (1 ml) was centrifuged at 10,000 rpm for 1 min. The supernatant was collected to determine the protein content and activity of enzymes.

Determination of protein content

Protein content was assayed by method of Bradford (1976). Twenty microliters of enzyme extraction were mixed with 3 ml of protein reagent. The test tubes were shaken for 5 minutes. The absorbance was measured at 595 nm.

Superoxide dismutase analysis

SOD activity was assayed by measuring its ability to inhibit the photochemical reduction of NBT using the method of Beauchamp, Fridovich (1971). The reaction mixture contained 1.8 ml of 50 mM potassium phosphate buffer pH 7.8, 50 µl of 16 mM EDTA, 50 µl of 0.4 M methionine, 20 µl of 1 mM riboflavin and 50 µl of crude enzyme extract. Control set was prepared according to above except replacing crude enzyme with 50 µl of distilled water. The test tubes were exposed to fluorescence light for 30 min and the absorbance was measured at 560 nm.

Catalase activity analysis

CAT activity was assayed by measuring the rate of disappearance of H_2O_2 using the method described by Chandlee, Scandalios (1984). The reaction mixture contained 1.8 ml of 50 mM potassium phosphate buffer pH 7.0, 100 µl of 0.5 M H_2O_2 were added to 100 µl of sample. The decrease in H_2O_2 was followed as a decline in absorbance at 240 nm.

Ascorbate peroxidase analysis

The activity was assayed by method of Whitaker (1994). The reaction mixture contained 1.7 ml of 50 mM potassium phosphate buffer pH 7.0, 50 µl of 0.5 M H_2O_2, 100 µl of 20 mM ascorbic acid, 50 µl of 16 mM EDTA and 100 µl of enzyme extract. The oxidation of ascorbate was followed by the decrease in the absorbance at 290 nm.

Guaiacol peroxidase (GPX) analysis

The activity was assayed by method of Nakano, Asada (1981). The reaction mixture contained 1.8 ml of 50 mM potassium phosphate buffer pH 7.8, 200 µl of 0.5 M H_2O_2, 200 µl of 3% guaiacol and 100 µl of enzyme extract. The disappearance of guaicaol was followed by the decrease in the absorbance at 472 nm.

Statistical analysis

Data was analyzed by using SPSS version 19.0. Means of each treatment were compared by using one-way ANOVA followed by Duncan's multiple range test. The significant level was separated at $p < 0.05$.

Results

Under drought stress, RWC was significantly decreased when compared to control group. All priming treatment slightly increased RWC compared to hydropriming. SPM priming significantly increased RWC content compared to hydropriming while other treatments were not significantly difference (Fig. 1a). In drought condition plants usually accumulate the compatible solutes such as amino acids, proteins, soluble sugars or proline. The similar trends were observed in this study. Proline content was significantly increased under drought condition while a small amount of proline was observed in control group. All of priming treatments, seedling showed proline accumulation under drought stress. Likewise, highest proline accumulation were significantly observed only in PEG and ASA priming (Fig. 1b).

Drought stress slightly decreased SOD activity in hydropriming treatment. Seed priming with PEG, SPM and CC significantly increased SOD activity under drought stress compared to hydropriming while other priming treatments had no effect on SOD activity (Fig. 2a). CAT activity significantly increased under drought stress compared to control group. Similar trend was observed in ASA, GB and CC priming compared to hydropriming. Conversely, activity of CAT in SPM treatment was as well as control group while slightly decreased activity was found in PEG priming compared to hydropriming (Fig. 2b).

The APX activity under drought condition was increased compared to control group. Seed priming with PEG, SPM, ASA and GB had no effect on APX activity compared to hydropriming while significantly increased APX was only observed in CC priming (Fig. 2c). On the other hand, our resulted showed that activity of GPX under drought stress had no significance compared to control. According to priming treatments slightly increased GPX activity was observed in SPM, ASA, GB and CC priming compared to hydropriming although the decreased of GPX activity was observed in PEG priming but also no significant difference compared to hydropriming.

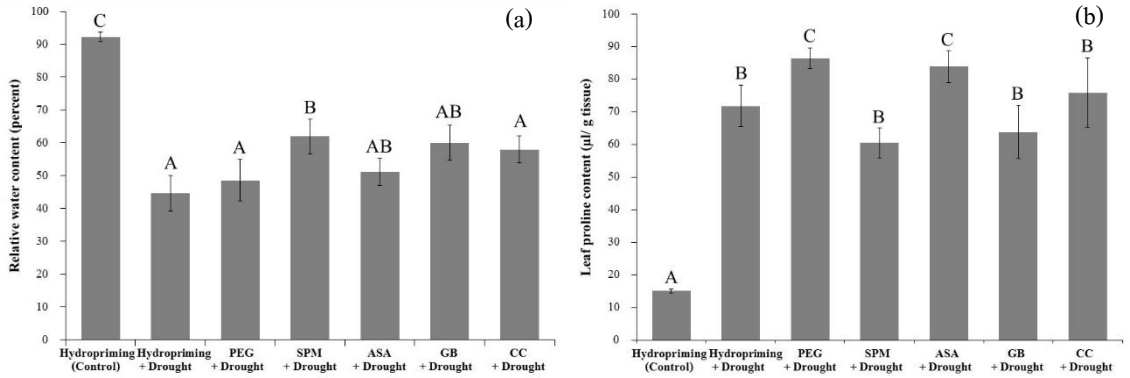

Figure 1 Effects of difference seed priming methods on (a) RWC content and (b) proline content of *O.sativa* cv. KDML 105 rice seedlings after induced drought stress condition with 15% of PEG for 10 days. The data represented the mean ± SE (n = 5). The Duncan's multiple range was tested at $P < 0.05$ level of significance.

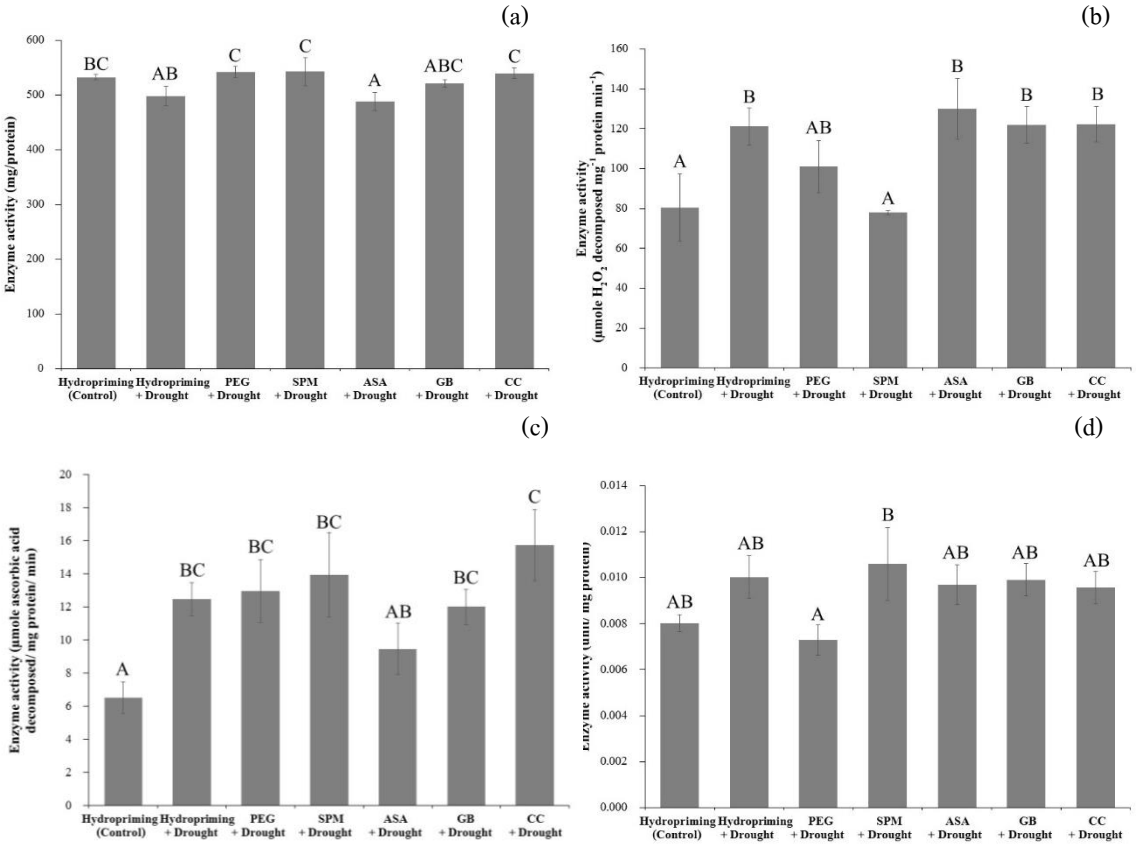

Figure 2 Effects of seed priming on the activities of antioxidant enzymes (a) SOD (b) CAT (c) APX and (d) GPX of *O.sativa* cv. KDML 105 rice seedlings after induced drought stress condition with 15% of PEG for 10 days. The data represented the mean ± SE (n = 5). The Duncan's multiple range was tested at $P < 0.05$ level of significance.

Discussion and Conclusions

Drought stress, an important abiotic factor, was limited rice seedlings growth by reduced water uptake results in a-decreased water content in plant tissue. As a results, turgor pressure was lost and then plants growth was inhibited. Seed priming is a technique which used to improved germination rate and uniformity. The mechanism of seed priming is to initiate the metabolic system while radicle postulation is prevented. Moreover, stress tolerance mechanism may be activated during priming process (Chen, Aurora, 2013). In this study, all priming improved RWC when compared to hydropriming and significantly increased RWC was observed only in SPM priming. Due to SPM property, it has been reported that SPM may be involved in maintaining membrane integrity and membrane stability (An et al., 2012; Pál et al., 2015). Likewise, proline accumulation is a common phenomenon observed in plant while subjected to environmental stress. In plants, intracellular proline level have been found to increase during exposure to various stresses e.g. drought, UV radiation, heavy metal, pathogen and oxidative stress. The function of proline in stress condition is play a role as an osmolyte and pressure balance during stress. Moreover, proline also acts as a chemical chaperone in plant cell. Due to this properties, proline have also been proposed to involved in the stabilization of proteins and antioxidant enzymes (Liang et al., 2013). In this study, proline content was increased under drought stress when compared to control group. Seed priming with PEG and ASA significantly increased proline content compared to hydropriming. The results are supported by Moghanibashi et al. (2013) who reported that free proline content were enhanced by priming with PEG in sunflower during germination. According to study of Farooq et al. (2013) who reported that two cultivars of wheat seeds, including Mairaj-2008 and Lasani-2008, primed with ASA significantly increased proline accumulation under drought stress. The effect of seed priming related to proline mechanism needs to be further investigated.

Environmental stress causes oxidative stress by production and accumulation of ROS. These molecules can be controlled by antioxidants and antioxidant enzymes. Enzymatic antioxidant system is an important role to counteracts the effect of ROS. Overall this study indicated that activities of antioxidant enzymes were higher in all priming treatment compared to hydropriming. Antioxidant enzymes including SOD, CAT, APX and GPX are known to be effective against oxidative stress. These enzymes regulate lipid peroxidation and cell membrane integrity by balancing and scavenging the ROS. This study indicated that better RWC and proline accumulation in rice seedlings might be effect from seed priming accompanied by increased activity of antioxidant enzymes. Moreover, several studied were reported that seed priming with these agents including ASA, GB and CC can increase the activities of antioxidant enzymes in sorghum, wheat and aromatic rice (Zhang et al., 2015; Farooq et al., 2013; Farooq et al., 2008). Interestingly, the activities of antioxidant enzyme including SOD and APX were lower observed in ASA priming compared to hydropriming. This effect might due to function of ASA which also acts as a strong antioxidant in plants. Application of exogenous ASA by seed priming may thus increase endogenous ASA level that could help in balancing of ROS in early rice seedlings. The results showed that, SOD and APX activity were lower than other treatment.

In conclusion, from our results indicated that seed priming has a potentially to improve drought tolerance. This response might due to effect of priming agents could leave as a priming memory in seed during priming process. As a consequent, seed priming is one method that introduced to increase drought tolerance of rice seedlings cv. KDML 105.

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