Promotive Effect of Priming with 5-Aminolevulinic Acid on Seed Germination Capacity, Seedling Growth and Antioxidant Enzyme Activity in Rice Subjected to Accelerated Ageing Treatment

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Abstract: Seed deterioration by ageing caused by natural and artificial treatments lowers germinability and viability. Seed priming is a technique used to relieve seed deterioration from ageing. We evaluated the effect of seed priming with 5-aminolevulinic acid (ALA) at various concentrations on the seed germination capacity, seedling growth, antioxidant enzyme activities, and lipid peroxidation in the rice seedling cultivar Pathum Thani 1 with or without exposure to artificial ageing treatment. Seeds were subjected to accelerated ageing treatment by exposure to high relative humidity and high temperature conditions for 84 hr (aged seeds). The shoot length was significantly reduced and the injury index and H2O2 formation were increased in the seedlings emerged from the aged seeds. Priming of the aged seeds with 0.5 μg mL⁻¹ ALA significantly enhanced the seed germination capacity and seedling relative growth rate to values higher than those obtained by priming with water (hydropriming). However, there was no significant difference in superoxide dismutase, peroxidase and ascorbate peroxidase activity as compared with hydropriming. In the seeds not given ageing treatment priming with ALA significantly increased the superoxide dismutase and peroxidase activities in the seedlings by 144% and 282%, respectively, as compared with the hydropriming treatment. Seed priming with ALA at 0.2 μg mL⁻¹ significantly increased the relative growth rate of seedling and lowered seedling water content as compared with the values without priming or with hydropriming. Seed priming with ALA was confirmed to enhance rice seed germination capacity, seedling growth, and antioxidant enzyme activity in rice seedlings.

Key words: Ageing, 5-Aminolevulinic acid, Antioxidant enzyme activities, Germination, Lipid peroxidation, Rice, Seed priming.

Rice seeds undergo natural or artificial ageing during long-term storage which leads to deterioration in seed quality and viability. The loss in seed viability and longevity consequently causes extended emergence time, non-uniform emergence, poor crop stands, and low crop yields (Rajjou and Debeaujon, 2008). Accelerated ageing of most crop seeds, which consists of seed storage under conditions of high humidity and temperature, causes severe damage and a loss of vigor in the seeds. This is associated with a progressive decrease in seed germination (Sveinsdóttir et al., 2009). Accelerated ageing was developed as a test method to estimate the longevity of seeds under a range of storage conditions (Ravikumar et al., 2002). Processes employed during seed storage have created seed aging and have caused free-radical-mediated lipid peroxidation, enzyme inactivation or protein degradation, disruption of...
cellular membranes, and damaged genetic material such as nucleic acids (McDonald, 1999; Murthy et al., 2003) which lead to poor germination. Seed priming, a pre-sowing hydration technique, is a strategy to improve the seed germination capacity at suboptimal temperatures and in hypoxia (Smok et al., 1993) and to increase the reinvigoration of aged seeds (Bailly et al., 1998). The germination capacity of primed aged seeds has been related to the ability of antioxidant enzymes to scavenge reactive oxygen species (ROS), the superoxide radical (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), and hydroxyl radicals (OH$^-$) (Bailly et al., 2002). Hence, in an antioxidant activity system, superoxide dismutase (SOD), which converts O$_2^-$ to H$_2$O$_2$, and ascorbate peroxidase (APX) which eliminates H$_2$O$_2$ in the ascorbate-glutathione cycle (Asada, 1992), play important roles in the control of ROS levels during imbibition and germination in the seed cells (Bailly, 2004).

Improvement of seed performance by hormonal treatment, which includes plant growth regulators during seed priming and other pre-sowing seed treatments, are well documented (Jisha et al., 2013). 5-Aminolevulinic acid (ALA) is an aliphatic precursor in the biosynthesis of all porphyrin compounds such as chlorophyll, heme, and phytochrome (Wang et al., 2004). ALA is a biodegradable herbicide and insecticide, but it has promotional effects on the growth and photosynthesis of crops and vegetables, and is harmless to humans, animals, and crops (Sasaki et al., 1993; Hotta et al., 1997a 1997b). The compound presumably promoted the chilling tolerance effect in plants under low-light which suppressed respiration (Hotta et al., 1998; Wang et al., 2004), and improved tolerance to salinity stresses (Nishihara et al., 2003). ALA not only promotes the growth and yield of crop plants, but also acts as an alleviator of oxidative damage and enhances the activity of antioxidant enzymes such as superoxide dismutase (SOD), peroxidase (POD), and ascorbate peroxidase (APX) to encounter radicals under stress conditions i.e., drought stress and NaCl stress (Nishihara et al., 2003; Li et al., 2011; Liu et al., 2011). Korkmaz and Korkmaz (2009) proposed that seed priming with ALA enhanced the germination rate of pepper seedlings under chilling stress, and improves germination, and seedling uniformity, especially under unfavorable environmental conditions. The action mechanism of ALA in promoting seed germination, antioxidant enzyme activity, and lipid peroxidation of plants exposed to accelerated ageing also needs to be fully elucidated. Application of ALA has been found to promote the activity of antioxidant enzymes under unfavorable environments. Therefore, seed priming with ALA might mitigate the seed ageing effects by enhancing the activity of antioxidant enzymes to scavenge ROS during rice seedling growth under accelerated ageing. Our hypothesis focused on whether seed priming with ALA could increase the germination capacity and antioxidant enzyme activity of the rice seedling cultivar, Pathum Thani 1. The present work addressed the effect of seed priming with ALA on the seed germination capacity which was linked to antioxidant enzyme activities and lipid peroxidation of the rice seeds subjected to accelerated ageing treatment. The objectives of the study were: (1) to study the germination capacity and seedling growth of the rice seeds generated by seed priming regimes; (2) to investigate the rice seed germination ability by determination of the enzyme activities involved in ROS scavenging; and (3) to determine the lipid peroxidation and H$_2$O$_2$ accumulation during the rice seed germination with and without accelerated ageing treatment.

Materials and Method

1. Seed materials and seed priming treatments

Seeds of ‘Pathum Thani 1’ paddy rice with an initial moisture content of 11.95% (dry weight basis), all from the same seed lot, were obtained from the Rice Research Center, Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom, Thailand. A sample of 7,200 seeds of the same size was carefully selected and weighed. The seeds were randomly divided into two groups of 3,600 seeds each, one group was subjected to ageing treatment where the seeds were exposed to 42°C at 100% relative humidity (RH) for 84 hr (Association of Official Seed Analysts, 1983) (aged rice), and the other was not treated (non-aged rice). Each group was further divided into 9 sub-groups of 400 seeds each for priming treatment. The seeds in each sub-group were randomly divided into 4 groups with 100 seeds each to provide 4 replications.

The aged and non-aged rice seeds were subjected to priming treatment, which consisted of soaking the seeds in aerated distilled water for 24 hr (hydropriming), soaking the seeds in a solution containing ALA at concentrations of 0.002, 0.004, 0.008, 0.015, 0.07, 0.2, or 0.5 μg mL$^{-1}$ in 100 mL for 24 hr or were not given priming treatment. The ratio of seed weight to solution volume was 1:5 (g mL$^{-1}$) according to Farooq et al. (2006). The ALA solution was prepared by dissolving 5-aminolevulinic acid (ALA) hydrochloride salt which was supplied by Sigma-Aldrich (Gillingham, UK) in distilled and deionized water to the desirable concentrations. The solution and water during seed priming were used at ambient temperature (26 ± 2°C). After seed priming, the primed seeds were air-dried in the shade to the initial moisture content.

2. Seed germination measurement

The experimental seeds in all treatment groups were sown on paper towels saturated with distilled water having pH 6.2 and conductivity 0.1 μS cm$^{-1}$ in 36 covered transparent polystyrene germination boxes (33 cm × 20 cm × 10 cm), 200 seeds (2 replications)/box with a partition for each replication. The seeds were placed in a
germination cabinet with an average temperature of 25°C for 14 d. The experiment was carried out using 4 replicates. Seeds with a primary root, shoot axis, cotyledon, and coleoptile were counted as germinants. The first and final counts were carried out on the 4th and 14th day of sowing, respectively (International Seed Testing Association 2006, 2007). The time to achieve 50% germination was calculated according to the formulae of Coolbear et al. (1984) modified by Farooq et al. (2005). The final germination percentage was calculated according to the equations of Ellis and Roberts (1981). The germination index was calculated as described in the Association of Official Seed Analysts (1983). All of the variables of germination were recorded on the 4th day after sowing, and at the time to achieve 50% germination.

3. Determination of injury index

The injury index was estimated using the seeds sown on paper towels as described above and measurement of the electrolyte leakage by the method reported by González-Aguilar et al. (2004). The rice seeds (1 g from each replication) obtained on the day of germination were washed with distilled water to remove the solution from the injured cells and immersed in 20 mL of 0.3 M mannitol solution in a 50 mL plastic tube at room temperature. The tubes were gently shaken for 3 hr and the electrical conductivity of each solution was measured using a conductometer (ATI Orion model 162, Thermo Scientific Corporation, Beverly, MA, USA) at 25ºC. The initial electrical conductivity was measured and then each tube was immersed in boiling water for 30 min and set aside until reaching room temperature. The final electrical conductivity was measured again in the resultant solution. The cell membrane stability or the injury index (I) was estimated from the formula as described by Kocheva et al. (2004):

\[ I = 1 - \frac{(1 - T_1/T_2)}{(1 - C_1/C_2)} \times 100, \]

where \( T_1 \) and \( T_2 \) are the first and second measurements of the electrical conductivity of the solutions in which treated samples are immersed and are taken prior to and after boiling, respectively, whereas \( C_1 \) and \( C_2 \) are the respective values for the controls.

4. Determination of \( \alpha \)-amylase activity and reducing sugar content

The activity of \( \alpha \)-amylase was determined as reported by Reiss (1994). The hull, roots, shoot, and scutellum of the seedlings obtained 5 days after germination (Palmiano et al., 1972) were removed and 1 g of the dehulled and degemermed grains from each replication was homogenized on ice with 30 mL of 10 mM citric acid-sodium citrate buffer at pH 5. The homogenate was centrifuged at 15,000×g for 10 min at 4°C, and the supernatant was collected to determine the enzymatic activity. The enzyme activity was determined from the supernatant by the starch-iodine method. Reaction assays were initiated by adding 2 mL 0.05% starch in 0.05 M citric acid-sodium citrate buffer (pH 5) and 1 mL of enzyme. After 5 min of incubation, 7 mL of 1 N HCl was added to stop the enzymatic reaction, followed by the addition of 1 mL of iodine reagent. Following color development, the iodine-treated sample was measured for absorbance at 580 nm. A reaction assay without the addition of enzyme was carried out as the control. A unit of the starch-iodine assay was defined as the disappearance of an average of 1 mg iodine-binding starch material per minute in the assay reaction.

The reducing sugar content was colorimetrically measured as described by Miller (1959). Reducing sugar was extracted by homogenizing 0.5 g of the dehulled and degemermed grains obtained 5 days after germination (Palmiano et al., 1972) from each replication with 5 mL of distilled water. The homogenate was centrifuged at 12,000×g for 10 min. The reducing sugar content was measured using 0.3 mL of supernatant diluted with 2.7 mL of distilled water and mixed with 3 mL of 1 3, and 5% dinitrosalicylate and 1 mL of 40% sodium potassium tartrate. The intensity of the color of the supernatant mixture was measured at 575 nm.

5. Determination of membrane lipid peroxidation

The malondialdehyde (MDA) content was measured as membrane lipid peroxidation according to the method of Hodges et al. (1999) using the thiobarbituric acid test. Young leaves obtained 12 days after sowing on paper towels as described above were homogenized in 40% trichloroacetic acid (TCA) containing 0.65% thiobarbituric acid (TBA). The extracts were centrifuged at 10,000×g for 15 min after incubation in a water bath at 95°C for 30 min and then immediately placed in an ice bath. The amount of MDA-TBA complex was calculated by its specific absorbency at 532 nm in supernatant, with the nonspecific absorbency at 600 nm being subtracted.

6. Measurement of \( \text{H}_2\text{O}_2 \) content

The \( \text{H}_2\text{O}_2 \) content was colorimetrically measured by the method reported by Velikova et al. (2000) using young leaves obtained 12 days after sowing on paper towels as described above. \( \text{H}_2\text{O}_2 \) was extracted by homogenizing 200 mg leaf tissue from each replication with 2 mL of 0.1% TCA. The homogenate was centrifuged at 12,000×g for 15 min. To determine the \( \text{H}_2\text{O}_2 \) level, 1 mL of the extracted solution was mixed with 1 mL of potassium phosphate buffer (10 mM, pH 7.8) and 2 mL of 1 M KI. The intensity of the yellow color of the supernatant mixture was measured at 390 nm.
7. Antioxidant enzyme activity assays

Approximately, 0.2 g of fresh leaf tissue from each replication obtained 12 days after sowing on paper towels as described above was homogenized on ice with 4 mg of polyvinylpolypyrrolidone (PVPP) and 2 mL of 100 mM sodium phosphate buffer (pH 7.8) including 10 mM EDTA, 10 mM phenylmethane-sulphonylfluoride (PMSF), and 5 mM ascorbic acid. The homogenate was centrifuged at 13,000×g for 15 min at 4°C, and the supernatant was collected to determine the enzymatic activity. The superoxide dismutase (SOD) activity was determined as described by Yu and Rengel (1999) in a 1 mL reaction mixture containing 50 mM Hepes buffer (pH 7.6), 0.1 mM EDTA, 50 mM Na2CO3, 13 mM methionine, 0.025% Triton-X 100, 75 μM nitroblue tetrazolium (NBT), 2 μM riboflavin, and 20 μL enzyme extract spectrophotometrically at 560 nm based on the photoreduction of NBT. The blue formazan produced by NBT photoreduction was measured by an increase in absorbance at 560 nm. An SOD unit was defined as the amount of enzyme required to inhibit 50% of the NBT photoreduction.

The peroxidase (POD) activity was determined based on an increase in absorbance at 430 nm as described by Radić et al. (2006). The mixture was composed of 1 mL reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0), 20 mM pyrogallol, 1 mM H2O2 and enzyme extract. A POD unit was defined as an increase in absorbance of 1.0 per minute using the extinction coefficient of 2.47 mM−1 cm−1.

The ascorbate peroxidase (APX) activity was determined according to Yamaguchi et al. (1995), by monitoring the oxidation rate of ascorbate at 290 nm. The mixture was composed of 1 mL reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0), 1.2% sucrose, 10 mM 3-aminotriazole, 0.5 mM ascorbate, 10 mM H2O2, and enzyme extract.

8. Seedling growth measurement

A new batch of 7,200 Pathum Thani 1 rice seeds was subjected to ageing and seed priming treatments as described above. However, the experimental seeds in each group (100 seeds each) were germinated by placing the rice seeds in a sand box (33 cm × 20 cm × 10 cm) containing approximately 2,500 g of cleaned sand for 21 days until the emergence of the rice seedling. The sand-growing medium was prepared by ensuring 90% of the sand particles passed through a 0.8 mm sieve. The sand was rinsed with tap water until the rinsed water was clean, followed by a final single rinse using distilled water. The sand growing media contained 8.08 mg kg−1 P, 66.4 mg kg−3 K, 21.9 mg kg−3 Fe, 0.0306 mg kg−1 Cu, 0.349 mg kg−3 Zn, 0.476 mg kg−3 Mn, 0.0775 mg kg−1 Mo, 0.561 mg kg−1 B, 0.543 mg kg−1 Ni, and 36.2 mg kg−3 Na. To minimize the effect of nutrient contamination from other sources and to confirm the promotional effect on rice seedling growth by the seed priming treatments, we added only deionized water to maintain the field capacity of the sand growing medium. The sand boxes were placed under ambient conditions where the average day/night temperature was 32.4/26.7°C, The RH was 59.7/77.0% and the average daily photosynthetic photon flux density (PPFD) ranged from 652 to 1,081 μmol PPF m−2 s−1.

The root and shoot lengths of the seedlings were recorded at 21 days after sowing. At 28 days after sowing, all seedlings were removed from the sand, washed with distilled water, and examined for seedling growth. The fresh weight (FW) was recorded after the removal of surface water by blotting and the dry weight (DW) was determined after drying for 15 min in an oven at 80°C followed by drying in a vacuum dryer at 40°C to constant weight. The relative growth rate was defined as (ln DW after treatment − ln DW before treatment) / treatment duration (Kingsbury and Epstein, 1984). The water content of the seedlings was calculated as: 100 × (FW − DW) / FW (Yang et al., 2008).

Data analysis

The experimental data were subjected to analysis of variance, and the differences among means were determined by Duncan’s multiple range test (SAS, 2003). Means differing at P ≤ 0.05 were considered significant.

Results

1. Seed germination capacity

There were no significant differences in the final germination percentage, the germination index and the time to achieve 50% germination between the aged seeds and the non-aged seeds without priming or those given hydropriming (hydroprimed) or primed with ALA at various concentrations, except for the hydroprimed aged seeds which required a significantly longer time to achieve 50% germination than the non-aged seeds (Fig. 1). However, the seed priming treatments had a significant effect on the seed germination capacity. Hydropriming and priming with ALA at various concentrations resulted in a significantly higher germination percentage from both aged and non-aged seeds than from seeds without priming treatment. Aged seeds primed with ALA at various concentrations required a significantly shorter time to achieve 50% germination than those with hydropriming (Fig. 1C). Priming with ALA at 0.015, 0.07, 0.2 and 0.5 μg mL−1 significantly increased the germination index of the aged seeds as compared with the hydropriming treatment. Priming of the aged seeds with ALA at 0.5 μg mL−1 significantly improved the final germination percentage and germination index, and significantly reduced the time to achieve 50% germination by 10.22, 40.87, and 43.44%, respectively, as compared with the hydropriming treatment (Fig. 1).
2. **α-Amylase activity and reducing sugar content**

There were no significant differences in the α-amylase activity between the seedlings from the aged and non-aged seeds without priming and those subjected to hydropriming or priming with ALA at various concentrations except for 0.002 and 0.008 μg mL⁻¹ which gave significantly lower α-amylase activity than that in the seedlings from non-aged seeds (Fig. 2A). However, the accelerated ageing treatment significantly reduced the reducing sugar content in the seedlings from the aged and non-aged seeds subjected to the priming treatments (Fig. 2B). The seedlings from the aged seeds showed no significant differences in the α-amylase activity and reducing sugar content with the seed priming treatment. Priming with ALA at 0.002 μg mL⁻¹ resulted in the highest α-amylase activity in seedlings from the non-aged seeds (Fig. 2A). Hydropriming and priming with ALA at 0.002
and 0.008 μg mL⁻¹ significantly increased the reducing sugar content in the seedlings from the non-aged seeds by 38.50, 42.46 and 37.51%, respectively.

3. Antioxidant enzyme activities

The SOD and POD activities in the seedlings showed no significant differences between the aged and non-aged seeds with the seed priming treatments except that the seedlings from the aged seeds primed with ALA at 0.5 g mL⁻¹ had significantly lower SOD and POD activities than those from the non-aged seeds (Fig. 3A-B). Accelerated ageing tended to reduce the POD activity by 76.49 – 528.91%. There were no significant differences in the SOD, POD and APX activities with the seed priming treatments in the aged seeds. However, seed priming with 0.5 μg mL⁻¹ ALA significantly increased the activities of SOD and POD in the leaves from the non-aged seeds than hydropriming or no priming (Fig. 3A-B). There were no significant differences in the APX activity between aged and non-aged seeds hydroprimed or primed with ALA at various concentrations except that the non-aged rice seeds primed with ALA at 0.015 g mL⁻¹ had a significantly higher APX activity than that of the aged seeds (Fig. 3C). Priming of the non-aged seeds with ALA at 0.015 μg mL⁻¹ significantly increased the APX activity to be higher than in the non-priming and hydropriming by 197.90 and 141.01%, respectively (Fig. 3C).

4. Lipid peroxidation, H₂O₂ content and injury index

At 12 days after sowing, aged seeds either hydroprimed or primed with ALA at various concentrations tended to have MDA content in the rice seedlings higher than that of the non-aged seeds although the differences were not significant. However, the aged seeds given hydropriming treatment had a significantly higher MDA content in the rice seedlings than the non-aged seed (Fig. 4A). Both hydropriming and priming with ALA at various concentrations tended to reduce the MDA content in the seedlings compared to the non-primed seeds but the differences were not significant, except for the rice seedlings from the aged seeds primed with 0.2 μg mL⁻¹ which had a significantly lower MDA content than those from the non-primed seeds. There were no significant differences in the H₂O₂ content among the seedlings from the aged or non-aged seeds hydroprimed or primed with ALA at various concentrations except for the seeds from the aged seeds primed with ALA at 0.002, 0.008 and 0.2 μg mL⁻¹ which had a significantly higher H₂O₂ content than those from the non-aged rice seeds (Fig. 4B). Seedlings from the aged seeds primed with ALA at 0.004 and 0.015 μg mL⁻¹ had significantly lower H₂O₂ content in the leaves than those of the non-primed seeds (Fig. 4B). The seedlings from the aged seeds showed a significantly higher injury index irrespective of seed priming treatment.
except for ALA at 0.004 μg mL⁻¹ and hydropriming. Treatment of both aged and non-aged seed with ALA at various concentrations and hydropriming significantly reduced the injury index as compared with the seeds without priming (Fig. 4C).

5. Seedling growth

There were no significant differences in the relative growth rate, seedling WC and root length between the seedlings from the aged and non-aged seeds without priming treatment (Fig. 5A, 5B and 5D). However, the seedlings from the non-aged seeds had a significantly higher shoot length than that the seedlings from the aged seeds under non-priming conditions (Fig. 5C). There were no significant differences in the relative growth rate of the rice seedlings between those from the aged and non-aged seeds with the priming treatment, except for the seedlings from the aged seeds treated with ALA 0.2 μg mL⁻¹ which had a significantly lower relative growth rate than those from the non-aged seeds, in contrast to those primed with ALA at 0.004 μg mL⁻¹ which had a significantly higher relative growth rate than the seedlings from the non-aged seeds (Fig. 5A). Seedlings from the aged seeds not primed and hydroprimed had a significantly lower relative growth rate than those from the aged seeds primed with ALA at 0.004 μg mL⁻¹ which had a significantly higher relative growth rate than the seedlings from the non-aged seeds (Fig. 5A). There was no variation in relative growth rate of the seedlings from the aged seeds primed with ALA at various concentrations. Aged seeds primed with ALA at 0.004 μg mL⁻¹ gave a potentially higher seedling relative growth rate than the non-aged seeds. There was no significance difference in the seedling water content in the seedlings from the aged seeds with the seed priming treatment. However, the non-aged seed primed with ALA at 0.002 μg mL⁻¹ gave the lowest seedling water content (Fig. 5B). There were no significant differences in shoot length between the seedlings obtained from aged and non-aged rice seeds subjected to the priming treatments except for the seeds primed with ALA 0.002 μg mL⁻¹ and non-primed (Fig. 5C). The seedlings from the aged seeds hydroprimed or primed with ALA priming at various concentrations had no significant difference in root length from those from the non-primed seeds, but the seedlings from the non-aged seeds primed with ALA at 0.004, 0.008, 0.015 and 0.2 μg mL⁻¹ had significantly longer root lengths than those from the non-primed rice seeds (Fig. 5D). Aged seeds hydroprimed or primed with ALA at all concentrations except 0.002 μg mL⁻¹ gave significantly longer shoot lengths than the non-primed seeds (Fig. 5C). In addition, rice seedlings from the non-aged rice seeds primed with ALA at 0.004, 0.008 and 0.2 μg mL⁻¹ had significantly longer root lengths than those from the aged seeds. Seedlings from the non-aged

Fig. 4. Effects of ALA seed priming on: A) MDA content; B) H₂O₂ content, and C) injury index of rice seedling during germination under accelerated ageing and non-ageing. Values are the means of 4 replicates. Means followed by different letters in the same graph are significantly different at P < 0.05, according to Duncan’s multiple range test. NP = Non-priming, HP = Hydropriming.
Discussion

Generally, aged seeds have physiological mechanisms responsible for the slow root growth of the seedlings. Accelerated ageing treatment led to the deterioration of both germinability and seed viability (Kapoor et al., 2011). The artificial and natural aged seeds showed the first signs of physiological deterioration by the slower germination to the radical emergence stage and delayed radical emergence or a high mean germination time (Matthews et al., 2012). The slow metabolic repair at non-optimum temperatures may have resulted in an increase in the delay in the mean germination time of most deteriorated seeds because of a greater need for repair. However, there were no significant differences in the seed germination capacity between aged and non-aged seeds or priming treatments which might have been due to the high seedling vigor inherited from the genetic background of the rice variety (Wang et al., 2010). Our results suggested that rice seed priming with ALA at 0.5 μg mL⁻¹ could ameliorate the germination capacity even after exposure to the accelerated ageing treatment. Our findings are similar to those obtained by Korkmaz and Korkmaz (2009) who demonstrated that seed priming with ALA enhanced the germination rate, germination and seedling uniformity of pepper seedlings, especially under unfavorable environmental conditions of chilling stress. Our findings were also consistent with those obtained by Rae-hyun and Song (2007) who demonstrated that the application of Rhodopseudomonas sp. which produced indole-3-acetic acid and 5-aminolevulinic acid could increase the germination percentage of tomato seed by 30.2%. Due to the seed deterioration by accelerated ageing, the α-amylase activity in seedlings obtained from aged rice seeds tended to be lower than that in the seedlings from the non-aged seeds (Fig. 2). However, Sung and Jeng (1994) reported that the respiratory metabolism was also stimulated during accelerated ageing. This was accompanied by a reduction in the reducing sugar content in the rice seedlings (Fig. 2). In this process, the ROS was subsequently produced and would be degraded by the greater antioxidant enzyme activities induced by priming. The high respiratory rate resulted in the elevation of lipid peroxidation and H₂O₂ formation (Fig. 4).
Rice seedlings responded differently to priming with ALA at various concentrations, thereby resulting in different biochemical changes and germination ability after the accelerated ageing (Fig. 1 and 2). Seed priming with ALA at a high concentration of 0.5 μg mL^{-1} significantly elevated the SOD and POD activities in the rice seedlings from non-aged rice seeds, but not in the seedlings from aged rice seeds (Fig. 3). A number of studies have focused on the stimulation of seedling antioxidant enzymes by ALA treatment, including: SOD in spinach seedlings subjected to salinity stress (Nishihara et al., 2003); pakchoi seedlings grown under optimal conditions (Memon et al., 2009); APX, CAT and SOD in *Brassica napus* under water deficit stress (Liu et al., 2011); and CAT in soybean plants from the damaging effects of cold stress (Balestrasse et al., 2010). However, the results of the present study revealed that there were no significant differences in the SOD and POD activity between the seedlings of aged rice seeds primed with ALA at various concentrations and non-primed seeds. There are two possible explanations for this discrepancy. First, the ALA concentrations for seed priming employed in the current study (0.002 – 0.5 μg mL^{-1}) were lower than those in the other investigations. Korkmaz et al. (2010) reported that seed soaking with 25 ppm ALA providing chilling stress tolerance of pepper seedling, while Zhang et al. (2013) also demonstrated that the exogenous application of ALA at 25 mg L^{-1} significantly improved SOD, POD and CAT in seedling leaves of *Cassia obtusifolia* L. under salinity stress. The second possibility is that the scavenging property of the antioxidative enzymes depends on the concentration of ALA. Seed priming at a low ALA concentration employed in the study did not increase the SOD, POD and APX activities enough to completely detoxify the ROS produced during the accelerated ageing of the seed. SOD, POD and APX activities during the accelerated ageing should be measured in further studies to obtain a comprehensive explanation of its impact on rice seed germination after accelerated ageing. The present study also indicated that the ALA concentration cannot fully restore the SOD and POD activity in aged seed. It is possible that ageing had caused severe deterioration in the SOD and POD activity in the aged seed to an extent that the priming with ALA at various concentrations could not restore the enzyme activities. In addition, the ALA concentration employed in the study might not have been adequate to restore these enzyme activities. Further study is needed to examine the effect of ALA at higher concentrations on the enhancement of the antioxidant enzyme activities until full alleviation occurs of the ROS production in the accelerated aged rice seeds. Although there were no significant differences in the SOD, POD and APX activities with the priming treatment in the seedlings of the aged seeds, priming with ALA tended to increase the SOD and POD activities. Therefore, the improvement in the germination capacity may be attributable to the improvement in antioxidant enzyme activity. Moreover, seed priming with ALA especially at 0.004 μg mL^{-1} significantly promoted the seed germination capacity, seedling relative growth rate and shoot length more than without priming. This was surprising as there might be other mechanisms other than the antioxidant enzymes which regulate the growth rate of rice seedlings from aged rice seed primed with ALA. ALA is able not only to act as an antioxidant enzyme promoter, but also to stimulate nitrate reductase activity (Beyzaei et al., 2014), and the respiration rate and ATP synthesis (Fu et al., 2014). The effects of rice seed priming on the activities of mechanisms other than antioxidant enzyme activity should be further studied.

H₂O₂ is utilized by POD in the oxidation of various inorganic and organic substrates (Asada, 1994). The results of the present study demonstrated that although rice seed was exposed to accelerated ageing, seed priming with ALA at 0.004 and 0.015 μg mL^{-1} significantly reduced H₂O₂ formation in the seedlings when compared to the non-priming treatment. The seedlings from the aged rice

![Graph A](image1)

![Graph B](image2)

**Fig. 6.** Correlation between: A) SOD activity and H₂O₂ formation; and B) POD activity and H₂O₂ formation in rice seedlings.
seeds primed with ALA at 0.008 and 0.015 μg mL⁻¹ tended to have a higher SOD activity than those from the aged seeds without priming treatment. Therefore, the enhancement of the SOD and POD activity by ALA reduced the H₂O₂ content in the rice seedlings. Among the seed priming treatments, accelerated ageing without priming had a pronounced effect on the activities of ROS scavenging enzymes in rice leaves during seedling growth (Fig. 3) which was due to a marked decrease in the POD activity (Fig. 3B). ALA at all concentrations examined except 0.002 μg mL⁻¹ tended to eliminate the effects of the ROS production due to the accelerated ageing by a detoxification mechanism (Fig. 4) that included an increase in the antioxidant enzyme activity of SOD, and POD. Fig. 6 shows the negative relationship between antioxidant enzyme activities and H₂O₂ formation found in this study. The ROS detoxification potential of the seed can be restored by the seed priming treatments, and the invigoration treatment of seeds is controlled by imbibition ultimately improving their vigor (Rajjou and Debeaujon, 2008).

Membranes are susceptible to damage resulting from accelerated ageing (Bailly et al., 1998). Malondialdehyde (MDA), which is derived from the final decomposition product of lipid peroxidation, has been used as an index for lipid peroxidation (Goel et al., 2003). In the present study, the MDA content of the rice seeds was increased after the accelerated ageing treatment. Priming with ALA at various concentrations had no effect on the MDA content in the rice seedlings from the aged rice seeds, but ALA 0.2 μg mL⁻¹ priming significantly decreased the MDA content in rice seedlings from the aged rice seeds. These results indicated that the MDA content in the rice seedlings had a negative relationship with the ALA concentration used in the seed priming treatments (Fig. 7). An increase in the ALA concentration for seed priming resulted in a lower MDA content in the rice seedlings. However, this finding was in contrast to that reported by Zhang et al. (2008) who found that the MDA content had a positive relationship with ALA treatment. This was probably due to the concentration of ALA employed in the experiment of Zhang et al. (2008) was much higher than in our study (0.2 vs. 50 μg mL⁻¹, respectively), and ALA applied at a high concentration could act as a herbicide (Sasikala et al., 1994). The data of the present study also indicated that rice seed priming with ALA at a concentration of 0.2 μg mL⁻¹ produced no harmful effects on the seedlings although the seed had been subjected to accelerated ageing treatment.

Seed priming with 0.2 μg mL⁻¹ ALA significantly improved the seedling relative growth rate and root length, whilst decreasing the seedling WC (Fig. 5). Our results indicated that rice seed primed with 0.2 μg mL⁻¹ ALA had the potential to increase cell division and elongation in the seedlings. ALA stimulated photosynthesis and thus more cellular protein synthesis in the plants (Hotta et al., 1997b; Zhang et al., 2013). Seedlings with a higher relative growth rate will have a denser cellular content, i.e., cell organelles, and thus lower water content of the cells. The higher activity in cellular physiological activities resulted in more energy being utilized and a lower reducing sugar content in the plants (Fig. 2). Seed priming of non-aged rice with ALA at 0.004, 0.008, 0.015 and 0.2 μg mL⁻¹ significantly enhanced the root length of rice seedlings as compared with the non-priming and hydropriming treatments. Gray and Steckel (1983) concluded that priming increased the embryo length, which resulted in the early initiation of germination in carrot seeds. Germination enhancement has been suggested to be a consequence of DNA, RNA and protein synthesis involved in the repair of the membrane and enzyme during hydration treatments (Farooq et al., 2007). ALA not only promoted the germination capacity but also increased the embryo length of rice seedlings from non-aged rice.

In conclusion, although there were no significant differences in the antioxidant enzyme activity and α-amylase activity among seedlings of aged seeds subjected to the priming treatments, seed priming with ALA at 0.5 μg mL⁻¹ significantly promoted the seed germination capacity, seedling growth and antioxidant enzyme activity in the non-aged rice seeds. Thus, it is useful to prime rice seed with ALA to improve the seed germination capacity and seedling growth for rice production. It is necessary to investigate the effect of ALA at higher concentrations on the antioxidant enzyme activity in order to fully elucidate the mechanism of ALA as a promoter of these enzymes.

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