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Germination and Biochemical Changes in Accelerated Aged and Osmoprimed *Pinus thunbergii* Seeds

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Abstract : The aim of this study was to investigate relationship among seed viability and enzymes activities involved in scavenging reactive oxygen species (ROS), especially, superoxide dismutase (SOD), glutathione reductase (GR), and catalase (CAT). In other respects, osmopriming has been demonstrated to reinvigorate aged seeds. Various viabilities of seeds that were ranged from 80 to 100% of germination rate could be produced using osmopriming and accelerated aging treatments. Priming treatment of *Pinus thunbergii* seeds for 3 days at 15°C with a polyethylene glycol solution at -1.2 MPa improved their subsequent germination at 25°C. Accelerated aging (3, 6, 9, and 12 days at 41°C and 100% relative humidity) decreased seed germination percentage depending on aging treatment duration. Electrolyte conductivities of seeds were measured as assay of membrane integrity. The conductivity from electrolyte leakage of *P. thunbergii* seed was also correlated with seed germinability. Conductivity for control seeds that had 95% of germination percentage was 3.48 μS g^{-1}, but jumped as doubled (7.98 μS g^{-1}) in 12-day-aged seed that had 80% of germination percentage. Our results demonstrate that aging of *P. thunbergii* seeds is associated with changes in the electrolyte leakage, lipid peroxidation, and antioxidant defense system. Priming of aged seeds progressively restored the initial germinative ability and resulted in a marked decrease in the levels of MDA and conductivity of seed leachate. These effects of priming were also well recovered of GR and CAT activities in aged seed. The improved seed quality by priming treatment appears at least partly attributable to reduced lipid peroxidation, resulting from enhanced antioxidative enzyme activities that are suggesting the antioxidant defense systems play a key role in seed vigor.

Key words **:** *accelerated aging, Pinus thunbergii, electrolyte leakage, germination, lipid peroxidation, antioxidative enzyme, priming*

Introduction

Seeds during storage lost their ability to germinate. Lipid peroxidation, enzyme inactivation, protein degradation, and disruption of cellular membranes were reported as major causes (McDonald, 1999a; Narayana Murthy *et al*., 2003; Ratajczak and Pukacka, 2005; Pukacka and Ratajczak, 2007). Accelerated aging conditions such as high temperature and high seed water moisture lead to biochemical deterioration during seed aging (McDonald, 1999a). In these cases, lipid peroxidation and the loss of membrane phospholipids are major cause of seed aging under accelerated aging conditions; the consequence of formation of an increase amount of free oxygen radicals (Goel and Sheoran, 2003).

The electrical conductivity (EC) test is acknowledged as one of the best tests for the evaluation of the loss of cell membrane integrity by the concentration of electrolytes released by seeds during imbibition. Cell membrane integrity is considered one of the primary physiological events of seed deterioration process (Matthews and Powell, 2006). There were several reports on the increase of EC in relation of seed vigor loss in pine seeds (Demelash *et al*., 2004; Singh and Bonner, 2001; Tammela *et al*., 2000) and beech seeds (Pukacka and Ratajczak. 2007).

The major cause of the loss of membrane integrity is believed to be the peroxidation of unsaturated fatty acid, which may take place with participation of reactive oxygen species (ROS) (McDonald, 1999a). Lipid peroxidation starts with the generation of free radicals and peroxides. The produced free radicals and peroxides may cause injury to cellular components like membrane lipids, proteins and nucleic acids during quiescence and germination states. In fresh seeds, free radicals and peroxides are generally kept at low levels by cooperative reactions of antioxidant enzymes (Chang and Sung, 1998 and McDonald, 1999a). If the production of free radicals

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and peroxides exceeds the rate of their removal, seeds become oxidatively stressed and seed viability is reduced. Major ROS-scavenging enzymes include superoxide dismutase (SOD), glutathione reductatse (GR) and catalase (CAT). SOD is a key enzyme in the regulation of the amount of superoxide radicals and peroxides. Hydrogen peroxide (H_2O_2) can react in the Haber-Weiss reaction forming hydroxyl radicals (Mittler *et al*., 2004) that cause lipid peroxidation. CAT is implicated in the removal of H_2O_2. The removal of H_2O_2 through a series of reactions is known as an ascorbate- glutathione cycle in which ascorbate and glutathione participate in a cyclic transfer of reducing equivalents resulting in the reduction of H_2O_2 to water (H_2O) using electrons derived form nicotinamide adenine dinucleotide phosphate (NADPH) (Goel and Sheoran, 2003). In the present study, membrane integrity and lipid peroxidation were measured using EC and malondialdehyde (MDA), respectively. Levels of MDA and activities of antioxidant enzymes in aged seed were analyzed to see their relationship.

Priming of seeds is used extensively to improve germination performance. Through priming, seed deterioration caused by lipid peroxidation is prevented via greater dehydrogenase activity and lower peroxide formation (McDonald, 1999b). Moreover, increase in various free radical scavenging enzymes, such as superoxide dismutase, catalase and peroxidase have also been reported (Bailly *et al*., 1998; Chiu *et al*, 2006; Jeng and Sung, 1994). The role of antioxidant enzymes in priming processes are investigated for artificially aged seed and subsequent primed seed in aged seeds at the present time.

The native species, *Pinus thunbergii*, which survives on the seaside, was mainly recommended for rehabilitation of reclaimed coastal lands with extreme environmental conditions (Kwon *et al*., 2004). Long-term storage of pine seeds is essential for silvicultural purposes, especially in Korea, where good seed years are rare. Therefore information about germination of pine seed depending on seed aging has significant economic value.

The aim of the present study were (1) to characterize the sensitivity of *P. thunbergii* seed to aging conditions, and (2) to investigate whether seed deterioration is associated with electrolyte leakage, lipid peroxidation, and antioxidative enzyme activities in aging or subsequent priming treated seed. For this purpose, *P. thunbergii* seeds were artificially aged at 41°C at 100% RH and subsequently osmoprimed.

Materials and Methods

1. Plant material

Seeds were collected in 2005 from the seed orchard in Jeju Island operated by Forest Research Institute, Korea. The seeds were extracted, cleaned, and stored in darkness and at -18°C until use in 2006. Before the storage, damaged and insect infected seeds were discarded, and the empty seeds were eliminated using the water floating method.

2. Germination test

Germination tests were performed on two layers of filter paper saturated with water in 9-cm-diameter glass petri dishes. Four 50-seed replicates for each treatment were used. Experiments were carried out in a temperature-controlled growth chamber at 25°C under constant light. Germination count was performed daily for 28 days. The seed was considered as germinated if the radicle protruded 2 mm or longer from the seed coat following the ISTA rules (ISTA, 2006).

The parameters gathered include: germination percentage, mean germination time (MGT) and germination value (GV). MGT was calculated using the formula: $MGT=n_id_i/n$, where n is the total number of germinated seeds during the germination test, n_i is the number of germinated seeds on each day d_i and i is the number of days during the germination period (between 0 and 28 days) (Youshseng and Sziklai, 1985). GV is the product of peak value (PV) and average daily germination (MDG) (Czabator, 1962) while PV is the quotient of the highest value of the cumulative germination percentage, divided by the number of days from the beginning of the test.

3. Artificial accelerated aging

For the artificial accelerated aging (AA) tests, seeds were placed on a wire mesh screen and suspended over 100 mL of water inside a plastic accelerated aging box (11 cm×11 cm×4 cm). Boxes were placed at 41°C and 100% relative humidity for 3, 6, 9, and 12 days (coded as AA3, AA6, AA9 and AA12, respectively) in an incubator. After this aging period, seeds were used for germination tests, PEG priming treatment, and electrical conductivity, lipid peroxidation, and antioxidant enzymes.

4. PEG priming treatment

Seeds were primed using osmotic solutions at -0.4 and -1.2 MPa for three days in darkness at 15°C using polyethylene glycol (PEG 8000). The water potential of the PEG solution for osmopriming treatments was determined using the calculation method of Michel (1983).

5. Electrical conductivity

The electrical conductivity of the leachate from whole imbibed seeds was determined on four replications of 10 seeds (0.1 g) soaked in 50 mL distilled water for 24 hours at 24°C. The electrical conductivity of leachates in leaked solution was measured using conductivity meter

(CONSORT Model C533, Belgium).

6. Lipid peroxidation

Lipid peroxidation was determined by measuring the amount of malondialdehyde (MDA) produced by thiobarbituric acid reaction as described by Heath and Packer (1968). Seeds (weighed 0.1 g prior to imbibition) imbibed for three days, were homogenized in 5 mL of 62.5 mM phosphate buffer (pH 7.8). The crude extract was mixed with the same volume of 0.5% (w/v) thiobarbituric acid solution containing 20% (w/v) trichloroacetic acid. The mixture was heated at 95°C for 30 min and then quickly cooled in an ice-bath. The mixture was centrifuged at 3000×g for 10 min and the absorbance of the supernatant was monitored at 532 and 600 nm using UV-120 (SHIMADZU, Japan). After subtracting the non-specific absorbance (600 nm), MDA concentration was determined by its molar extinction coefficient (155 mM^{-1} cm^{-1}) and the results expressed as mol MDA g^{-1} FW.

7. Antioxidative enzyme activities

Seeds imbibed for 3 days (0.1 g prior to imbibition) were homogenized under ice-cold condition with 5 mL of 50 mM phosphate buffer (pH 7.0), 10 mM ascorbic acid (AsA) and 1.0% (w/v) polyvinylpyrrolidone. The homogenate was centrifuged at 20,000×g for 30 min, and the supernatant was collected for enzyme assays. Superoxide dismutase (SOD) was assayed on the basis of the inhibition of reduction of nitro-blue tetrazolium in the presence of xanthine at 530 nm according to the method of Beauchamp and Fridovich (1971). Activity of glutathione reductase (GR) was assayed as described in Carlberg and Mannervik (1985). The assay was carried out in a reaction mixture containing 50 mM phosphate buffer (pH 7.8), 0.1 mM NADPH, 0.5 mM GSSH and 0.1 mL enzyme extract. The change in A_{340} was recorded for 5 min after the addition of enzyme extract. Catalase (CAT) activity was determined by following a two-step procedure (Fossati *et al*., 1980). The rate of dismutation of H_2O_2 to H_2O and molecular oxygen is proportional to the concentration of catalase. Samples containing catalase were then incubated in the presence of a known concentration of H_2O_2. After one minute incubation, the reaction was quenched with sodium azide. The amount of H_2O_2 remaining in the reaction mixture was then determined by the oxidative coupling reaction of 4-aminophenazone (4-aminoantipyrene) and 3,5-dichloro-2-hydroxybenzenesulfonic acid (DHBS) in the presence of H_2O_2 and catalyzed by horseradish peroxidase (HRP). The resulting quinoneimine dye was measured at 520 nm. All the activities of enzyme were measured using UV-120 (SHIMADZU, Japan).

8. Statistical Analysis

The data were statistically analyzed using Analysis of Variance of a Completely Randomized Design. Means were compared using the Duncan multiple range test (DMRT) at the 5% level. Statistical analyses were done using SAS System for Windows, Version 8.01 (SAS Institute, USA).

Results

Germination percentage and germination value (GV) of the seeds decreased with artificial aging treatment (Figure 1A). Final germination percentage reduced after 12 days of aging. The longer the treatment, the greater was the reduction of seed viability. GV showed greater

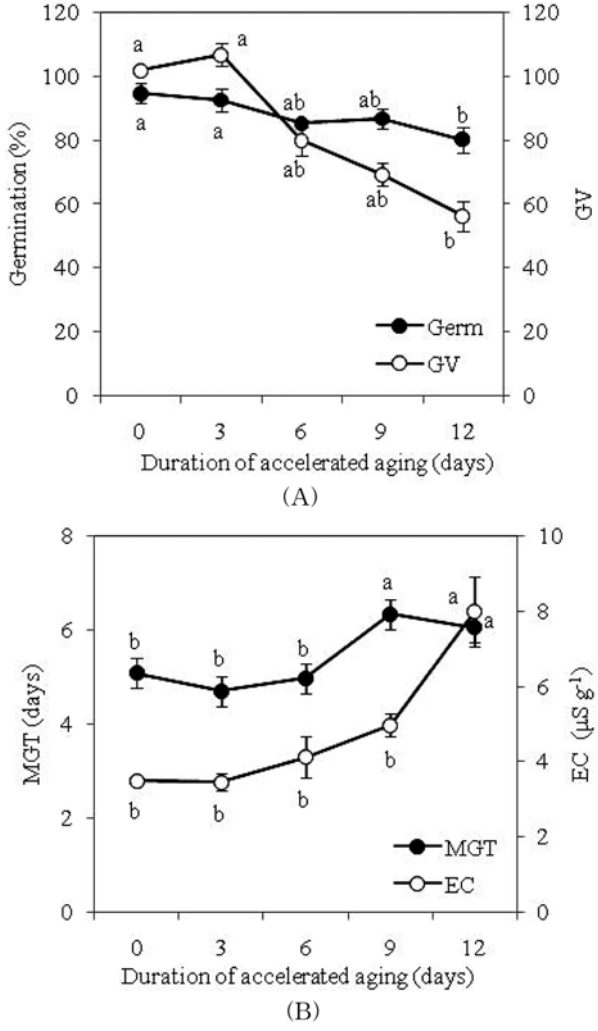

Figure 1. Germination percentage, germination value (GV) (A), and mean germination time (MGT), electrolyte leakage (EC) (B) after 3, 6, 9, 12 days of accelerated aging (AA) treatment at 41°C and 100% RH in *Pinus thunbergii* seeds. Values presented are mean±SE (n=4). The different letters are significantly different among accelerated aging duration at p < 0.05, Duncan's Multiple Range Test.

reduction (101 to 56) of seed viability than germination percentage (95 to 80%). GV was dramatically decreased after 6 days of aging even though germination percentage did not show significant changes. Electrical conductivity (EC) and mean germination time (MGT) increased progressively with the aging treatment (Figure 1B). Level of EC in control seed was 3.48 μS g^{-1}. After 12 days of aging, EC of *P. thunbergii* seeds increased to 7.98 μS g^{-1} that is twice higher than the control non-aged seeds. The mean MGT of artificial aging treated seeds for 12 days were about 1 day longer than that of control seeds.

The activities of superoxide dismutase (SOD) and glutathione reductase (GR) decreased with the aging duration (Figure 2A). SOD activity decreased from 0 to 6 days of aging, and it ranged from 297 to 222 unit g^{-1}. From 7 to 12 days, SOD was maintained from 222 to 224 unit g^{-1}. GR activity remained constantly until 6 days of aging and then decreased reaching 3164 and 2770 nmol g^{-1} at 9 and 12 days of aging, respectively. Contrary to SOD and GR activities, catalase (CAT) activity did not show a significant change in statistically, but it decreased with the aging duration, and it ranged from 762 to 710 unit g^{-1}. By contrast, MDA content increased markedly from 11.2 to 16.7 μmol g^{-1} with the increase of aging duration, i.e. the longer the duration of the treatment, the higher was their MDA content (Figure 2B).

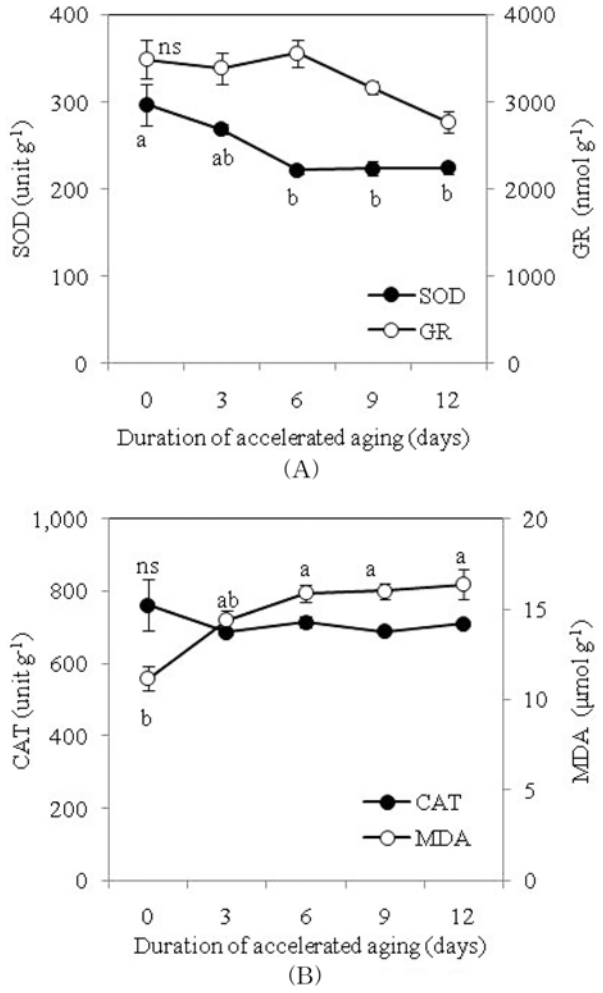

Figure 2. Changes in activities of superoxide dismutase (SOD), glutathione reductase (GR) and catalase (CAT), and malondialdehyde (MDA) content in artificial aged *Pinus thunbergii* seeds. Values presented are mean±SE (n=5). The different letters are significantly different among accelerated aging duration at p<0.05, Duncan's Multiple Range Test. ns: not significant.

PEG priming at -1.2 MPa (PEG 1.2) improved the germination, MGT, and GV as compared with the control. Aging treated seeds for 3 and 6 days could be recovered by 5.6, 27.5 and 56.9% after PEG priming at -1.2 MPa in germination percentage, MGT, and GV, as compared to the controls, respectively. PEG priming treatments were effective in decreasing electrolyte conductivity (EC) of seed leachates, which shows membrane stability. However, PEG priming treatment for aging treated seeds did not show any difference in EC. Among comparison of all the seed treatments, the lowest EC of seed leachate was observed in seeds primed with -0.4 MPa (PEG 0.4) and -1.2 MPa (PEG 1.2) PEG treatments (Table 1).

Table 2 represents the changes of antioxidant enzyme activity and MDA content in aging and priming treated seeds. The activity of SOD was decreased by accelerated aging treatment. Especially, priming treatment resulted in a decrease of SOD activity. There was no significant difference for all of the seed treatments in terms of GR activity. However, the activities of GR and CAT exhib-

Table 1. Germination percentage, mean germination time (MGT), germination value (GV) and electrolyte conductivity (EC) of PEG primed and accelerated aging treated *Pinus thunbergii* seeds at 41°C and 100% RH.

Treatment	Germination (%)	MGT (days)	GV	EC (μS g^{-1})
Control	94.7±3.1^{a}	5.1±0.2^{a}	102±1.1^{c}	5.8±0.19^{ab}
PEG 0.4	93.3±3.1^{ab}	3.6±0.2^{c}	150±21.5^{ab}	4.2±0.70^{b}
PEG 1.2	100.0±0.0^{a}	3.7±0.0^{c}	160±2.5^{ab}	4.6±0.50^{b}
AA3	92.6±3.6^{ab}	4.7±0.1^{ab}	107±3.5^{c}	5.7±0.37^{ab}
AA3+PEG 1.2	98.7±1.2^{a}	4.5±0.1^{b}	120±3.0^{bc}	5.8±0.11^{ab}
AA6	85.1±1.3^{b}	5.0±0.1^{ab}	80±5.0^{c}	6.8±0.91^{a}
AA6+PEG 1.2	93.3±3.1^{ab}	3.1±0.2^{d}	165±20.6^{a}	7.2±0.19^{a}

All values are means of four replicates ± standard error (SE). Values in the same column with different letters are significantly different from each other at p<0.05, Duncan's Multiple Range Test. PEG 0.4: primed at PEG solution of -0.4 MPa, PEG 1.2: primed at PEG solution of -1.2 MPa, AA3: accelerated aging treated for 3 days, AA3+PEG 1.2: primed at PEG solution of -1.2 MPa for 3 days of aged seed, AA6: accelerated aging treated for 6 days, AA6+PEG 1.2 : primed at PEG solution of -1.2 MPa for 6 days of aged seed.

Table 2. Changes of superoxide dismutase (SOD), glutathione reductase (GR) and catalase (CAT) activities, and MDA content of PEG primed and accelerated aging treated Pinus thunbergii seeds at 41°C and 100% RH.

Treatment	SOD (unit g^{-1})	GR (nmol g^{-1})	CAT (unit g^{-1})	MDA (μmol g^{-1})
Control	297±24^{a}	3489±219^{ns}	762±70^{bc}	11.2±0.7^{bc}
PEG 0.4	276±16^{ab}	4164±113	1441±51^{a}	10.9±0.3^{c}
PEG 1.2	243±13^{ab}	4171±119	1454±50^{a}	11.4±0.2^{bc}
AA3	269± 6^{ab}	3389±179	686± 7^{cd}	14.4±0.5^{a}
AA3+PEG 1.2	201± 7^{bc}	4200±109	523±47^{d}	13.3±0.7^{abc}
AA6	222± 4^{abc}	3561±154	715±15^{cd}	15.9±0.5^{a}
AA6+PEG 1.2	156± 3^{c}	3975± 59	954± 8^{b}	14.0±1.0^{ab}

All the values are means of five replicates ± standard error (SE). Values in the same column with different letters are significantly different from each other at $p < 0.05$, Duncan's Multiple Range Test. ns: not significant. PEG 0.4: primed at PEG solution of -0.4 MPa, PEG 1.2: primed at PEG solution of -1.2 MPa, AA3: accelerated aging treated for 3days, AA3+PEG 1.2: primed at PEG solution of -1.2 MPa for 3 days of aged seed, AA6: accelerated aging treated for 6 days, AA6+PEG 1.2 : primed at PEG solution of -1.2 MPa for 6 days of aged seed.

ited similar tendencies, i.e., GR and CAT activities increased with priming treatment. The activity of CAT was decreased by accelerated aging treatment. However, PEG primed seeds showed dramatic increase of CAT activities. The MDA content of PEG primed seeds exhibited different tendencies with enzyme activities after PEG priming treatment. The MDA content of PEG primed seeds was significantly lower than that of control and aging treated seeds ($P<0.05$).

Discussion

Aging of seeds leads to changes in seed quality, such as decrease in seed vigor followed by a loss of seed viability as previously shown by McDonald (1999a) and Lehner *et al*. (2008). As expected, the accelerated aging of *P. thunbergii* seeds carried out at 41°C and 100% RH lost their ability to germinate according to duration of aging treatment (Figure 1). In our experiment, germination was relatively high even after 12 days of aging treatment. Whereas, germination value (GV) and mean germination time (MGT) showed more clearly decrease of viability (Figure 1). The decline in germination percentage and an increase in mean germination time with accelerated aging were observed in the present study in accordance with previous studies on *Pinus strobus* (Singh and Bonner, 2001), *Pinus patula* (Demelash *et al*., 2004), *Pinus taeda* and *Pinus roxburghii* (Vanangamudi *et al*, 1998), and *Pinus rigida* seed (Han *et al*., 2006).

The first process during seed deterioration due to aging is excessive leakage of cytoplasmic components as a result of loss of membrane integrity (Smith and Berjak, 1995). This is clearly evident from the increased leachate conductivity value with aging in this study (Figure 1). Our study, thus, supports the utility of leachate conductivity as a routine supplement or a quick alternative to assess the germination capacity (viability) and the mean germination time (vigour) of pine seeds as suggested by other authors (Bonner, 1991; Rehman, *et al*. 1999; Demelash *et al*., 2004; Singh and Bonner, 2001).

The main enzymes involved in cell detoxification are superoxide dismutase (SOD), glutathione reductase (GR), catalase (CAT) (Bailly, 2004; Mittler, 2002). The ability of seeds to scavenge ROS is considered to be involved in seed storability (Bailly, 2004). In addition, aging is associated with a depletion of antioxidant enzyme activities in cotton (Goel *et al*., 2003), beech (Pukacka and Ratajczak, 2007) and sunflower seeds (Bailly *et al*., 1998). Lipid peroxidation has been demonstrated to occur during aging in Norway maple (Pukacka, 1991), beech (Pukacka and Ratajczak, 2007), *Pinus sylvestris* (Tammela *et al*., 2000) and sunflower seeds (Bailly *et al*., 1998). Our data re-confirmed these earlier findings. In our study, SOD, GR, and CAT activities decreased and MDA content increased according to duration of aging treatment. The results presented here with pine seed show that loss of seed viability is related to the increase of lipid peroxidation evaluated by MDA content (Figure 2).

Priming can reverse some of the sub-optimal temperature-induced deteriorative events, and thus improve seed performance (McDonald, 1999b; Chiu *et al*., 2006; Kim *et al*., 2008). Priming-enhanced seed performances are related to the repair and the build up of nucleic acid, enhanced synthesis of protein and repair of membranes (McDonald, 1999b). In this study, we found that PEG priming were significantly increased germination ability in aged seed (Table 1). These results were taken in conjunction with the improved germination and GV and shortened MGT. Enhanced antioxidative systems also contribute to improve quality in primed seed (Chiu *et al*., 2006). Priming enhanced CAT activities in our study. Especially, the increase of GR and CAT activities in primed seed for 6-day-aged seed supports priming effects such as the recover of seed deterioration. And also priming treatment decreased MDA content for aging treated seed (Table 2). The enzymes involved in the antioxidant defense system exhibit different sensitivities to aging depending on the species and the conditions of storage. In wheat seeds, SOD was more sensitive than CAT to a treatment at 45°C and 100% RH (Lehner *et al*., 2008), whereas in sunflower seeds placed in the same conditions CAT activity was more sensitive

than that of GR and SOD being almost unchanged (Bailly *et al*., 1996). Alteration of SOD activity was also observed during aging of seeds of *Acer platanoides* (Pukacka, 1991). The decrease of SOD activity of 6-day-aged seeds with priming did not reflect enhanced viability. However, the increase of GR and CAT activities was obvious in primed seeds in our results. Chiu *et al*. (2006) reported that priming produced high germinability and decreased MDA. The total peroxide accumulation could explain why priming improves the viability and vigor in seeds. It was also observed that the increased MDA content by aging treatment decreased with subsequent priming treatments.

Conclusion

Our results demonstrate that aging of *P. thunbergii* seeds is associated with changes in the electrolyte leakage, lipid peroxidation, and antioxidant defense system. There was a clear relation between increased electrolyte leakage with seed aging. Hence we suggest the leachate conductivity test as a routine supplemental test or as a quick alternative test to predict the viability of *P. thunbergii* seed. The improved seed quality by priming treatment appears at least partly attributable to reduced lipid peroxidation, resulting from enhanced anti-oxidative activities. The improved germination and emergence responses might be also related to the priming-enhanced activities of GR and CAT enzymes. Accelerated aging procedure could be a good indicator for understanding various metabolic changes associated with seed deterioration. However, accelerated aging cannot be used as the only model to understand the mechanisms involved in seed aging. Further investigations are required for natural aging conditions.

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