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Differences of Antioxidative Level in the Leaves and Seed Germinability among Five Natural Populations of *Hibiscus hamabo*

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Abstract - Variations in antioxidant capacity of leaves and characteristics of seed germination among five *Hibiscus hamabo* populations in Korea were evaluated. While the leaves of *H. hamabo* from Shincheon contained highest Na concentration, those from Hado and Ohzo does lowest. MDA content and SOD activity didn't show significant difference among populations, but total protein and anthocyanin content were significantly different among five populations. The filled seed rates of five populations were ranged from 92.0 to 98.7%, and the moisture contents of seeds from Shinyang and Soando were lower than that of H. syriacus (5.18%). The fresh weights of *H. hamabo* from Hado and Ohzo were the highest and those of seeds from Shinyang and Soando were the lowest. Significant variations among *H. hamabo* populations were detected for all seed germination characters: percentage of germination, average germination time and germination rate and uniformity. SOD activities of seeds from Onpyoung and Soando were the highest of five populations, and total protein concentrations of seeds in Ohzo and Shinyang populations were higher than those of other populations. *H. hamabo* is considered exposed to salt stress and all characteristics of seed germinations were inferior to other species.

Key words - *Hibiscus hamabo*, Antioxidative capacity, Seed germination, MDA, SOD

Introduction

Genus *Hibiscus* (Malvaceae) is a heterogeneous array of at least 250 species, most of which are tropical or subtropical (Wise and Menzel, 1971; Bates, 1965). This genus is classified twelve sections (Horchretiner, 1900) or thirteen series (Bates, 1965) based on morphological characteristics. The East Asian group, section Bombycella, includes *Hibiscus syriacus*, national flower of Korea, that is tolerant to low temperature. Most species in the genus about 200 species are distributed in tropical regions through temperate zones (references). Four *Hibiscus* species including *H. hamabo* are growing in (or are native to) Korea.

H. hamabo, native in Korea, is a deciduous shrub or sub tree of Malvaceae and reaches to 1 or 2 m in height. Pale yellow flowers bloom in August and its characters such as long flowering period are valuable for landscape. It is also expected to be used as a genetic source to improve the flower color of the Rose of Sharon.

H. hamabo has been strictly preserved by law of The Ministry of Environment and Korea Forest service, since the number of the species is very limited and could possibly be exterminated due to the habitat destruction and fragmentation. Protecting the natural habitats of the species may prevent the extinction. In order to establish the plan, studies on vegetation and environmental conditions in natural habitats must be preceded. In addition, the understanding of physiological characteristics of the species is essential for the establishment of effective and efficient conservation practices.

Ahn (2003) reported on the distribution of *H. hamabo* and ecological characteristics of natural habitats in Jeju Island. Ahn *et al.* (2003) also reported on vegetation and flora of *H. hamabo* in Soan Island to provide a preservation plan since *H. hamabo* is an endangered species and could possibly be exterminated in near future. Siepe *et al.* (1997) reported on the evaluation of genetic variability in a collection of *H. cannabinus* and *Hibiscus* species. In the respect of physiological studies, Huh and Kwack (1999) studied on the effects of uniconazole, gibberellin and calcium on salt injuries of *H. syriacus* and *H. hamabo* seedlings, and Warner and Erwin (2003) investigated on the effect of photoperiod and daily light integral on growth and flowering of five *Hibiscus* sp. Additionally, Omobuwajo *et al.* (2000) reported on the physical

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properties of sorrel (*H. sabdariffa*) seeds but the study on the seed characteristics of *Hibiscus* species was very few.

Also, the understanding on the seed characteristics is very important to make *in situ* or *ex situ* conservation strategy. Seed source testing of native species is desirable to screen the available variation for higher productivity and future breeding works. Selection of the best provenance of desired species for a given site or region is necessary for achieving maximum productivity in plantation forestry. Thus, the aims of the present study were to determine stress factors and antioxidant capacity to *H. hamabo* under natural populations and to examine the differences of seed germination and physiological characteristics among natural populations.

Materials and Methods

Leaf antioxidative characteristics

Plant samples were collected from at least randomly selected individuals of five populations (Hado, Ohzo, Onpyoung, Shinyang and Shincheon) in Jeju Island during June 2004. Collected leaf samples were carried to the laboratory in portable freezers (-20℃).

Lipid peroxidation in the leaves was determined by measuring the amount of malondialdehyde (MDA) produced by the thiobarbituric acid reaction as described by Heath and Packer (1968). 0.5g leaf sample was homogenized with 10ml of 62.5mM cold phosphate buffer (pH 7.8). The homogenate was centrifuged at 12,000 rpm for 20 min. The crude extract was mixed with the same volume of a 0.5% (w/v) thiobarbituric acid solution containing 10% (w/v) trichloroacetic acid. The mixture was heated at 95℃ for 15min 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 532nm and 600nm. After subtracting the non-specific absorbance (600nm), the MDA concentration was determined by its molar extinction coefficient ($155mM^{-1}$ cm^{-1}) and the results were expressed as mol MDA g^{-1} FW.

SOD activity and total protein content were analyzed. Fresh seeds (0.1g) were homogenized under ice-cold condition with 5ml of 50mM phosphate buffer (pH 7.0), 10mM ascorbic acid (AsA) and 1.0% (w/v) polyvinylpyrrolidone. The homogenate was centrifuged at 20,000 × g for 30min, and the supernatant was collected for enzyme assays. SOD was assayed based on the inhibition of reduction of nitro-blue tetrazolium in the presence of xanthine at 530nm according to the method of Beauchamp and Fridovich (1971). Protein content in the seeds was determined by the method of Lowry, using BSA as a standard protein (Lowry *et al.*, 1951).

For the analysis of anthocyanin, the sliced leaves (0.1g) were extracted for 4h in 10ml of 0.1% HCl-MeOH at room temperature. Absorption of the extracts at three wavelengths, 650, 620 and 530nm, were determined, and the absorbance of the anthocyanin extract was determined by means of the formula $(A_{530} - A_{620}) - 0.1(A_{650} - A_{620})$ (Proctor, 1974). The anthocyanin content was determined by using a molar extraction coefficient of 3.43×10^4 (Francis, 1982).

Dried leaves (0.5g each) were ground and used to determine Na content. Nitric acid (70%, 15ml) and hydrogen peroxide (30%, 5mL) were added to 0.5g of dried, ground seed sample in a digestion vessel. Samples were digested using the microwave digestion system, cooled after addition of distilled water, and filtered prior to analysis. Na content in the digested tissue was measured by atomic absorption spectrophotometer (AA-6701F, Shimadzu, Tokyo, Japan).

Seed characters and germinability

Seeds of *H. hamabo* were collected from at least randomly selected individuals of four populations (Hado, Ohzo, Onpyoung and Shinyang) in Jeju Island and one population in Soando, Jeonnam Province during the winter of 2003. Seeds of *H. syriacus* as a control were collected from Suwon, Kyounggi Province.

To evaluate the physical characteristics of seeds, the filled seed rate was determined after photographing the inner of the seed with X-ray (SOFTEX X-ray TV-25-1, Japan), and moisture content was expressed as a percentage (%) after measuring with infrared moisture balance (Precisa 310M, Swiss).

All germination tests were conducted under ISTA rules (International Seed Testing Association, 1985). Seeds were placed on three wetted Whatman No.3 filter paper discs in Petri dishes, each containing 50 seeds. All experiments were based on completely random designs with three replicates. Seeds were incubated in germination chamber in the dark condition at approximately 24℃. Seeds showing radicle emergences were recorded daily as 'germinated' and percent germination, mean germination time and germination rate and uniformity were calculated.

Percent germination (PG%) was calculated as the proportion of the germinated of the total number of viable seeds. Mean germination time (MGT), germination rate (GR) and germination uniformity (GU) were calculated as follows: MGT = $\Sigma(t_i n_i)/N$, GR = $\Sigma(n_i/t_i)$, and GU = $\Sigma[(MGT-t_i)^2 n_i]/N-1$, where t_i is the number of days counted from the beginning of the test, n_i is the number of seeds germinating on day *i* and N is the total number of germinated seeds.

Analyzing SOD activity and protein content of seeds followed the analyzing procedures for leaf samples.

The data were statistically analyzed using SAS for Windows Version 8.1 (SAS Institute Inc., Cary, NC, USA). Mean values per habitat were compared by ANOVA. When significant differences (P=0.05) were indicated, Duncan's multiple range tests were performed.

Results

Leaf antioxidative characteristics

Na concentration in the leaves showed significant difference among five populations (Fig. 1). Na concentration in the leaves represented the highest in the Shincheon population and the lowest on Hado and Ohzo populations.

MDA content was measured to evaluate physiological damage level. MDA content in leaves didn't show significant difference among five populations. In Table 1, MDA content was the highest in Onpyoung (726mol g^{-1}) and Shinyang (727mol g^{-1}) and the lowest in Ohzo (581mol g^{-1}). However total protein content in leaves was significantly different among populations. Total protein content was the highest in Shinyang (283g g^{-1}) and the lowest in Ohzo (135g g^{-1}). SOD activities was ranged from 357 unit g^{-1} to 458 unit g^{-1}, but didn't differ significantly among populations. SOD activity was the highest in the leaves in Shincheon (458unit g^{-1}) and the lowest in Hado (357unit g^{-1}). Anthocyanin, as a antioxidant, showed significant difference among five populations. The highest anthocyanin content was observed in the leaves of Hado (144ng g^{-1}), in contrast, the lowest content of anthocyanin represented in the leaves of Shincheon (77ng g^{-1}).

General properties of seeds

The filled seed rate, moisture content and fresh weight per 100 seeds of *H. hamabo* showed significant difference among five populations (Table 2). The filled seed rates of five populations were ranged from 92.0 to 98.7% and these values were higher than 74.7% of *H. syriacus*. The moisture contents of *H. hamabo* were from 3.23 to 8.35% and moisture contents of seeds from Shinyang and Soando were lower than that of *H. syriacus* (5.18%). In addition, the fresh weights per 100 seeds of *H. hamabo* from Hado and Ohzo were the highest and those of seeds from Shinyang and Soando were the lowest.

Germination properties

The percent germination, mean germination time and germination rate and uniformity of seeds of significantly differed among five populations (Table 3). The percent germinations of seeds from Hado, Ohzo, Onpyoung and Shinyang were from 45.3 to 50.7%, whereas percent germination of seed from Soando was 19.3% that is lower than that of *H. syriacus* (35.3%).

The range of mean germination time of *H. hamabo* was from 74.0 to 108.3 day, and the mean germination time of Shinyang

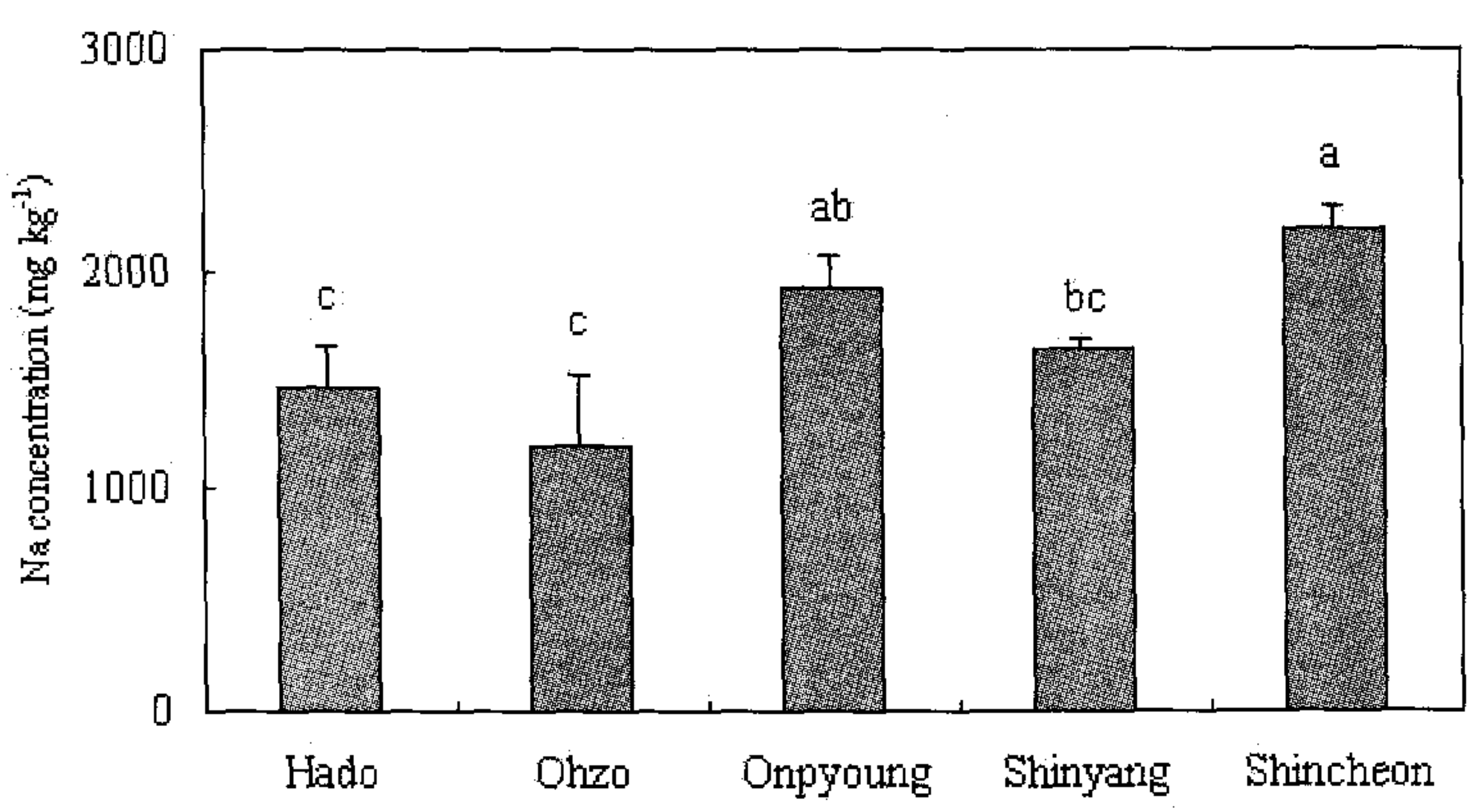

Fig. 1. Differences on Na concentration in the leaves of *H. hamabo* among five natural populations. Each bar represents the mean of five replicates standards deviation. Different letters indicate statistically significant differences among populations ($p < 0.05$).

(108.3 day) was the slowest among five populations. The mean germination time (16.3 day) of *H. syriacus* was faster than those of all tested *H. hamabo* populations. In addition, seeds from Shinyang and Soando represented slower germination rate than those of Hado, Ohzo and Onpyoung. That is, the germination rates of seeds in Shinyang and Soando were 0.17 and 0.08, respectively, and those of seeds in Hado, Ohzo and Onpyoung were 0.62, 0.43, and 0.52, respectively.

In case of germination uniformity, the seeds in Hado and Onpyoung represented 2562 and 2575, respectively, and the seeds in Ohzo, Shinyang and Soando were 1448, 1308, and 1461, respectively. Meanwhile, the germination rate of *H. syriacus* (1.33) was faster than those of *H. hamabo* and the germination uniformity of *H. syriacus* (35) was higher than those of *H. hamabo* as well.

SOD activity and total protein in seed

SOD activities in seeds didn't significantly differ among five populations but total protein concentrations in seeds were significant differences among five populations (Fig. 2). SOD activities in seeds of all tested *H. hamabo* were higher than that of *H. syriacus*, whereas total protein concentration was lower than those of *H. syriacus*. SOD activities of seeds from Onpyoung and Soando were the highest among five populations, and total protein concentrations of seeds from Ohzo and Shinyang populations were higher than those of other populations.

Discussion

Most wild *H. hamabo* are found at the road edge in the seacoast, and the area is thought to be the place where an environmental disturbance has been occurred severely. In addition, *H. hamabo* that is growing at the shore has been exposed various environmental stresses, such as strong sea wind, soil salinity, drought and low nutrients.

The values of Figure 1 and Table 1 showed Na concentration, physiological damage level and antioxidative characteristics in the

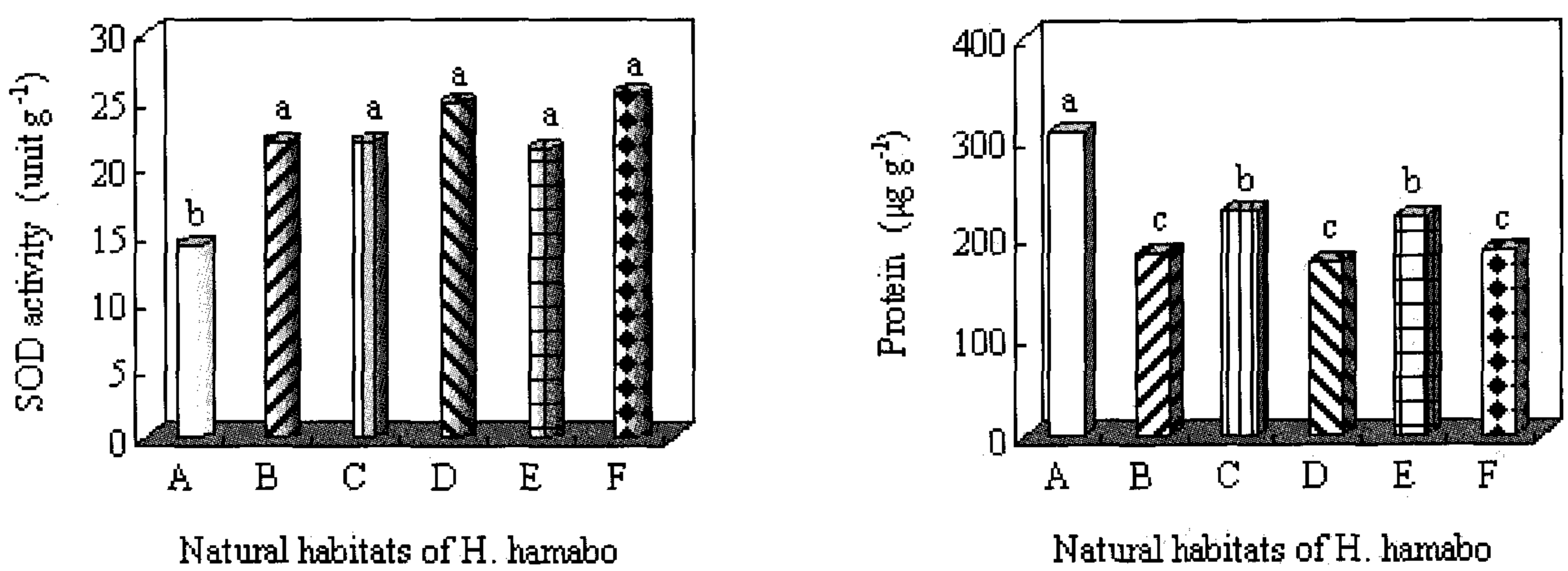

Fig. 2. Differences among natural populations on SOD activity and total protein concentration in seeds of *H. hamabo*. A: *H. syriacus* from Suwon as a control, B: *H. hamabo* from Hado Jeju, C: *H. hamabo* from Ohzo Jeju, D: *H. hamabo* from Onpyoung Jeju, E: *H. hamabo* from Shinyang Jeju, F: *H. hamabo* from Soando Jeonnam. Each bar represents the mean of three replicates. Different letters indicate statistically significant differences among populations ($p < 0.05$).

Table 1. Differences on MDA, protein, SOD activity and anthocyanin content in the leaves of *H. hamabo* among five natural populations

Habitat	MDA (μmol g^{-1})	Protein (μg g^{-1})	SOD (Unit g^{-1})	Anthocyanin (ng g^{-1})
Hado	680±100[a]	200±39[b]	357±76[a]	144±21[a]
Ohzo	581±148[a]	135±58[b]	363±58[a]	87±30[bc]
Onpyoung	726±69[a]	201±75[b]	426±113[a]	140±33[a]
Shinyang	727±151[a]	283±40[a]	418±76[a]	117±34[ab]
Shincheon	622±123[a]	143±48[b]	458±156[a]	77±21[c]
Pr > F	0.2723	0.0022	0.4872	0.0033

*Each data represents the mean of five replicates ± standards deviation. Different letters indicate statistically significant differences among populations ($p < 0.05$).

Table 2. Differences among natural populations on filled seed rate (FSR), moisture content (MC) and fresh weight per 100 seeds (FW) of *H. hamabo*

Species	Populations	FSR (%)	MC (%)	FW (g)
H. syriacus	Suwon	74.7±1.2[c]	5.18±1.40[b]	1.56±0.02[c]
H. hamabo	Jeju Hado	98.7±2.3[a]	8.35±1.22[a]	1.87±0.04[a]
〃	Jeju Ohzo	92.0±4.0[b]	8.30±1.22[a]	1.90±0.05[a]
〃	Jeju Onpyoung	93.3±3.1[ab]	5.03±1.64[b]	1.78±0.04[b]
〃	Jeju Shinyang	96.7±3.1[ab]	3.46±1.25[b]	1.59±0.01[c]
〃	Jeonnam Soando	93.3±4.2[ab]	3.23±0.17[b]	1.55±0.02[c]
Pr > F		0.0001	0.0001	0.0001

*Each data represents the mean of three replicates ± standards deviation. Different letters indicate statistically significant differences among populations ($p < 0.05$).

leaves of *H. hamabo* collected at five populations. Na concentration, protein and anthocyanin content were significant difference among populations, but MDA content and SOD activity were not significant difference among populations (Table 1). In general, *H. hamabo* was known as salt tolerant species. However, in our study, Na concentration in the leaves was correlated with SOD activity ($r = 0.92$), and the result suggests that *H. hamabo* is suffering from salt stress. In addition, Table 1 showed that stress strength and tolerance ability among populations significantly differed. In general, it has been reported that antioxidant capacity to stress varied from species to species and varieties even among cultivars of a single species (Öncel *et al.,* 2004; Calatayud and Barreno, 2004; Han *et al.,* 2006).

Meanwhile, a considerable variation in seed characters was observed among populations (Table 2). In special, moisture content and fresh weight varied significantly among populations. Most of variations observed in these characters are attributed to population variation (Mamo *et al.,* 2006), and variation in reproductive output (The total quantity of reproduction, such as flowers and fruits/seeds) has been shown to vary among populations, primarily due to differences in total and, to some extent, changes in resource allocation patterns (Bazzaz *et al.,* 2000). Under limited resources availability, a plant may allocate the available resource to the production of fewer larger seeds or many smaller ones (Harper *et al.,* 1970). However, plants under stressful environmental conditions (e.g. shade, drought and/or herbivory) favor selection for larger seeds, as they provide more reserves for successful establishment of seedlings (Moles and Westoby, 2004). The result is consistent with our result made on fresh weight. That is, the fresh weight of *H. hamabo* under stressful environmental condition was heavier than those of *H. syriacus* under favorable condition.

In addition, the germinations of seeds of *H. hamabo* collected from different sites varied significantly (Table 3). Mean germination time of *H. hamabo* seeds displayed most significant differences among populations, and the differences among populations of percent germination, mean germination time and germination rate were closely related to the differences of moisture content.

In most plant species, seeds vary in their degree of germination between and within populations and between and within individuals

Table 3. Differences among natural populations on percent germination (PG), mean germination time (MGT) and germination rate (GR) and uniformity (GU) of seeds of *H. hamabo*

Species	Habitat	PG (%)	MGT (day)	GR	GU
H. syriacus	Suwon	35.3±5.0[ab]	16.3±3.1[c]	1.33±0.44[a]	35±8[c]
H. hamabo	Jeju Hado	50.0±15.9[a]	81.7±8.9[ab]	0.62±0.25[b]	2562±825[a]
〃	Jeju Ohzo	47.3±10.1[a]	74.0±23.6[b]	0.43±0.07[bcd]	1448±671[b]
〃	Jeju Onpyoung	50.7±6.1[a]	78.7±4.0[ab]	0.52±0.02[bc]	2575±218[a]
〃	Jeju Shinyang	45.3±6.4[a]	108.3±11.9[a]	0.17±0.05[cd]	1308±679[b]
〃	Jeonnam Soando	19.3±14.0[b]	81.0±26.0[ab]	0.08±0.07[d]	1461±552[b]
Pr > F		0.0221	0.0003	0.0002	0.0014

*Each data represents the mean of three replicates ± standards deviation. Different letters indicate statistically significant differences among populations ($p < 0.05$).

(Benowicz et al., 2000, 2001; Gera *et al.,* 2000; Thomsen and Kjr, 2002; Sivakumar *et al.,* 2002; Mkonda *et al.,* 2003). Some of this variation can be of genetic origin, but much of it is known to be environmental, i.e. caused by the local conditions under which the seed matured.

In the present study, seeds of *H. hamabo* were exposed to salt stress. Soil salinity may affect the germination of seeds either by creating an osmotic potential external to the seed preventing water uptake, or through the toxic effects of Na^+ and Cl^- ions on the germinating seed (Khajeh-Hosseini *et al.,* 2003). Salt and osmotic stresses are responsible for both inhibition or delayed seed germination and seedling establishment (Almansouri *et al.,* 2001). Under these stresses there is a decrease in water uptake during imbibitions and furthermore salt stress may cause excessive uptake of ions (Murillo-Amador *et al.,* 2002). In many studies, seed germination and seedling growth were negatively affected by drought (Davidson and Chevalier, 1987; Kiem and Krostad, 1981; Owen, 1972; Passioura, 1988) and salinity stresses (Ashraf and Mcneily, 1988; El-Sharkawi and Salml, 1977; Francois *et al.,* 1986; Hampson and Simpson, 1990).

In our study, SOD activities in seeds of *H. hamabo* under stressful conditions were higher than that of *H. syriacus* under favorable conditions. The results suggest that *H. hamabo* was exposed to stronger stress than *H. syriacus*. Consequently, seeds of *H. hamabo* resulted the delayed mean germination time, slow germination rate, low germination uniformity.

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