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Effect of cold stratification, scarification and hormones on germination of dimorphic seeds of *Atriplex centralasiatica* under saline conditions

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Summary

The effect of cold stratification, seed coat scarification and plant hormones on germination of black and brown seeds of *Atriplex centralasiatica* under saline conditions was investigated. Cold stratification increased the germination rate, but did not affect final germination percentage of either brown or black seeds. Seed coat scarification and fluridone (Abscisic acid [ABA] biosynthesis inhibitor) improved germination of both types of seeds under salinity stress. In both brown and black seeds, gibberellin, 1-aminocy-cloproane-1-carboxylate (the immediate precursor of ethylene) and 6-benzyladenine, had little effect on the germination under salinity. Paclobutrazol (inhibitor of gibberellin biosynthesis) and ABA did not affect germination in distilled water, whereas ABA inhibited germination in saline conditions for both types of seeds. Paclobutrazol inhibited the germination of black seed only under salinity. Black seeds were more sensitive to ABA than brown seeds in salinity stress. Brown seeds contained more active gibberellins than black seeds, although they contained a similar level of ABA. In conclusion, the production of black and brown seeds showed bet-hedging ecological strategies. Gibberellins in seeds and ABA in seeds and bracteoles were the main plant hormones that affected the germination of dimorphic seeds.

Introduction

Atriplex centralasiatica, an herbaceous, salt-secreting halophyte in the family Chenopodiaceae, is widely distributed in China (Zhao, 2002). This species is a potential source of oilseed, food, high quality fodder and a tool for reclamation of saline soils (Zhao *et al*., 2002; Wang and Hou, 2005). In order to raise *A. centralasiatica* as a crop for economic purposes on saline soils, it is important to understand the seed dormancy/ germination characteristics of this plant. Recent reports showed that *A. centralasiatica* produces two types of seeds, brown and black seeds, and the difference in the two types of seeds was mainly in seed coat (Li *et al*., 2008a). The seed coat has been suggested to influence storability in some species, such as rapeseed (Zhang *et al*., 2006), *Suaeda*

salsa (Li *et al*., 2008b) and *S. aralocaspica* (Wang *et al*., 2008), because of differences in thickness and water absorbing ability.

The seed coat can also affect seed germination through inhibiting and mechanically restricting the embryo growth. Seed coat scarification might break seed dormancy and promote seed germination (Hermansen *et al*., 2000; Huang *et al*., 2004). However, in saline conditions, salinity might also damage the embryos of scarified seeds and affect seedling growth (Song *et al*., 2007). Salinity stress may also induce secondary dormancy of seeds. Whether scarification of the seed coat affects breaking of secondary dormancy of *A. centralasiatica* under salinity is unknown.

Plants of *A. centralasiatica* mainly grow in saline areas and salinity is the main factor that affects seed germination in its natural habitat. Some related studies have shown that hormones (like gibberellins, abscisic acid, ethylene, cytokinin) and cold stratification influence halophyte seed germination under salinity (Ungar, 1991; Khan and Ungar, 1997; Ali-Rachedi *et al*., 2004; Wang *et al*., 2008). A previous investigation showed that germination in black seed of *A. centralasiatica* was light sensitive in the presence of salinity: the germination of brown seed was sensitive to high temperature and the presence of bracteoles inhibited seed germination (Li *et al*., 2008a). No information is available on the effect of hormones and cold stratification on the germination of black and brown seeds under salinity. Light and temperature may affect biosynthesis of endogenous gibberellins (Yamaguchi and Kamiya, 2000) and bracteoles may contain ABA, which inhibits seed germination (Chen *et al*., 2007). Therefore, we hypothesize that gibberellins, abscisic acid, cytokinin and ethylene might play an important role in determining the germination of the different coloured seeds under salinity, because there are interactions among plant hormones, light, temperature and cold stratification in affecting dormancy and germination (Kucera *et al*., 2005).

There is little information about ecological and physiological indices of the two colours of seeds of *A. centralasiatica*, especially in relation to seed dormancy and germination. This investigation was therefore undertaken to study the effect of cold stratification, scarification and hormones on germination of black and brown seeds of *A. centralasiatica* under saline conditions.

Materials and methods

Seed collection and storage

Dispersal units of *A. centralasiatica* were collected in mid October, 2006 from coastal saline soils of Huanghua City, Hebei Province of north China. These dispersal units were air dried for a few days and stored in paper bags at room temperature.

Germination and seedling growth

The germination test was carried out in 2 ml 0, 60, 120, 240, 360 and 480 mM NaCl solution at 20/30°C in light (fluorescent lamp, intensity approximately 100 mmol m^{-2} s^{-1}) using triplicate samples. Seeds were washed with 0.02% Triton X solution, rinsed with distilled water, then washed with experimental solutions, and 50-100 seeds were placed

on two layers of wet filter paper (moistened with the experimental solutions as above) within a plastic Petri dish and closed. Seeds were scored as germinated when primary root protrusion was visible with a magnifier.

To assess seedling growth, germinated seeds were transferred to individual plastic pots (6 cm in diameter and 10 cm high) filled with perlite and placed in a growth chamber at a controlled temperature of 23°C, 40–60% relative humidity and continuous light (fluorescent lamp, intensity approximately 100 mmol m^{-2} s^{-1}).

Cold stratification

To determine the effect of cold stratification on seed germination, seeds were washed with 0.02% Triton X solution, rinsed with distilled water, then washed with experimental solutions (240, 360 and 480 mM NaCl). Three replicates of 50-100 of both black and brown seeds were put into Petri dishes lined with 2-layers of filter paper moistened with 2 ml experimental solutions and placed in the dark at 4°C for four days. After the cold treatments, seeds were transferred to 20/30°C in light (fluorescent lamp, intensity approximately 100 mmol m^{-2} s^{-1}). Seeds were scored as germinated when primary root protrusion was visible with a magnifier.

Scarification

The seed coats of brown and black seeds were carefully scarified (about 2 mm in length) with a scalpel on the lenticular side. The scarified seeds and intact seed (control) were incubated at the environmental conditions for germination described above.

Hormones and some hormonal biosynthesis inhibitors

For studying the effects of some plant hormones, gibberellin (GA_4), cytokinin (6-benzyladenine, BA), abscisic acid (ABA) and fluridone (Flu, ABA biosynthesis inhibitor) were added to give a final concentration of 10 μM during germination in 0, 240, 360, 480 mM NaCl solutions; ACC (1-aminocyclopropane-1- carboxylate, the immediate precursor of ethylene) was added to give a final concentration of 1mM and paclobutrazol (PAC, inhibitor of GA biosynthesis) was added to give 50 μM during germination in 0, 240, 360, 480 mM NaCl solutions.

Hormone analysis

For hormone analysis, three replicates of 500 mg dry brown/black seeds or flat/humped bracteoles were used. For cold stratification, 500 mg dry black/brown seeds were put into Petri dishes lined with 2-layers of filter paper previously moistened with 4 ml experimental solution and placed in dark at 4°C for four days. After cold treatments, seeds were transferred to 20/30°C in light for 24 hours. For salt treatment, 500 mg seeds were placed on filter paper moistened with either water (as control) or 360 mM NaCl at 20/30°C in light for 24 hours. Scarification was the same as previously described. After all treatments, seeds were harvested for hormone purification and analysis.

Purification of ABA: Samples of seeds (approximately 50 mg) were homogenized with 2 ml of 80% acetone: 20% water (v/v) through Tissue Lyser (Frequency at 30 s^{-1}, QIAGEN, Hilden, Germany) for ten minutes. Deuterium-labeled d_6-ABA purchased from

ICON SERVICES (Summit, NJ, USA) was used as internal standard. After adding 1 ng of internal standard, the homogenate was incubated in darkness for 12 h at 4°C and centrifuged at 3,000 g for 10 min at 4°C. The combined supernatant was dried under vacuum. Following re-suspension in 1 ml of 99% isopropanol: 1% acetic acid (v/v) by vortexing and sonication, samples were centrifuged (16,000 g for 5 min, 4°C), and the supernatant was transferred to a fresh tube and then dried again. Samples were dissolved in 50 ml of methanol and 450 ml of 1% acetic acid solution (v/v) was added. Oils in the samples were removed by partitioning using 1 ml of hexane, and following centrifugation (16,000 g for 5 min, 4°C); the remaining aqueous extracts were again removed to a fresh tube and dried by centrifugation under vacuum. Extracts were dissolved in 100 ml of methanol, and 900 ml of 1% acetic acid solution (v/v) was added. Oasis HLB 1 ml solid-phase extraction cartridges (Waters, Milford, MA, USA) were conditioned with 1 ml of acetonitrile followed by 1 ml of methanol and equilibrated with 1 ml of 1% acetic acid solution (v/v). Samples were loaded, followed by a wash with 1 ml of 1% acetic acid solution (v/v). ABA was eluted using 1 ml of 50% acetonitrile: 49% water: 1% acetic acid (v/v) before samples were dried under vacuum.

Purification of GAs: For GA purification, samples (approximately 500 mg) were homogenized in 15 ml acetone by homogenizer in a 50 ml centrifuge tubes. Deuterium-labeled d_2-GAs purchased from ICON SERVICES (Summit, NJ, USA) was used as internal standards. After adding 0.5 ng of internal standards, the homogenate was incubated in darkness for 12 h at 4°C (or 6 h at 20°C) and centrifuged at 3000 g for 20 min at 4°C. The combined supernatant was dried under N_2 gas. Following re-suspension in 15 ml of 50% acetonitrile : 50% water, oils in the samples were removed by partitioning using 15 ml of hexane for three times, and following centrifugation (3000 g for 20 min, 25°C), the remaining aqueous extracts were dried under N_2 gas to less than 2 ml solution left.

PVP (Polyvinylpyrrolideone) column: After PVP was washed by ten volumes of water three times, 3 ml mixture of PVP (water:PVP, 10:1 by weight) was added in 3 ml Bond-Elut reservoir (Varian) to elute water. Then 3 ml 100 mM K_2HPO_4 (pH = 8) was used to condition PVP column. Samples were dissolved in 0.5 ml 100 mM K_2HPO_4 (pH = 8) and loaded to PVP columns. After 2 ml 100 mM K_2HPO_4 (pH = 8) washed for two times, the eluted solutions were adjusted to pH = 2-3 by 6 N HCl.

HLB column: Oasis HLB 3 ml solid-phase extraction cartridges (Waters, Milford, MA, USA) were conditioned with 3 ml of acetonitrile followed by 3 ml of 2% formic acid solution (v/v). Samples were loaded, followed by two times wash with 3 ml of 2% formic acid solution (v/v). GAs was eluted using 3-4 ml of 80% acetonitrile: 2% formic acid (v/v) before samples were dried under vacuum.

DEA column: Varian 3 ml Bond Elut DEA cartridges were conditioned with 3 ml methanol. Samples were dissolved in 0.5 ml methanol and loaded, followed by a wash with 5 ml of methanol for two times. GAs was eluted using 5 ml 0.5% acetic acid in methanol for two times before samples were dried under vacuum.

Silicon column: Sep-Pak 1 ml Vac Silica cartridges (Waters, Milford, MA, USA) were conditioned with 1 ml chloroform. Samples were dissolved in 0.5-1 ml chloroform and loaded to column. GAs was eluted using 1 ml of 50% chloroform : 49% ethyl acetate : 1% formic acid (v/v) for two times before samples were dried under vacuum.

LC-MS/MS measurement: The resulting sample mixture was dissolved in 20 ml of water and 10 ml was injected. Endogenous ABA and GA levels were determined by LC-MS/MS analysis using a quadrupole/time-of-flight tandem mass spectrometer (Q-tof Premier; Waters) and an Acquity Ultra Performance liquid chromatograph (Waters) as detailed in Saika *et al.* (2007) and Varbanova *et al.* (2007). The amount of each compound was determined by spectrometer software (MassLynxTM v. 4.1, Micromass).

Data analysis

Seed germination was checked at two day intervals for 16 or 14 days. Rate of germination was calculated using a modified Timson's germination velocity index = $\sum$ G/t, where G = percentage of seed germinated after 16 days and t = total time of germination (Khan and Ungar 1984). Statistical analysis was done using Microsoft Excel statistical packages. Values of germination percentage were arcsine transformed before statistical analysis to ensure homogeneity of variance. The transformed values were analyzed using a two or three-way analysis of variance (ANOVA). If significant differences were found, Tukey's test was used to determine mean differences between treatments. All statistical analysis was done using SPSS 10.0 statistical software packages.

Results

Seed and seedling morphology of dimorphic seeds

There were two kinds of fruits from branches of *A. centralasiatica* plants, flat (figure 1 A) and humped fruits (figure 1 B). The ratio of flat: humped fruits varied from 1:1 to 2:1 for different individual plants. Both fruits contained brown or black seeds (figure 1 A and B). There were a few intermediate types (less than 1%). There was no significant difference in the embryo and endosperm of black and brown seeds; the only difference was in the seed coat colour. Seedlings of *A. centralasiatica* derived from black were much smaller (1.1 ± 0.1 cm in height) than those from brown seeds (2.4 ± 0.1cm in height).

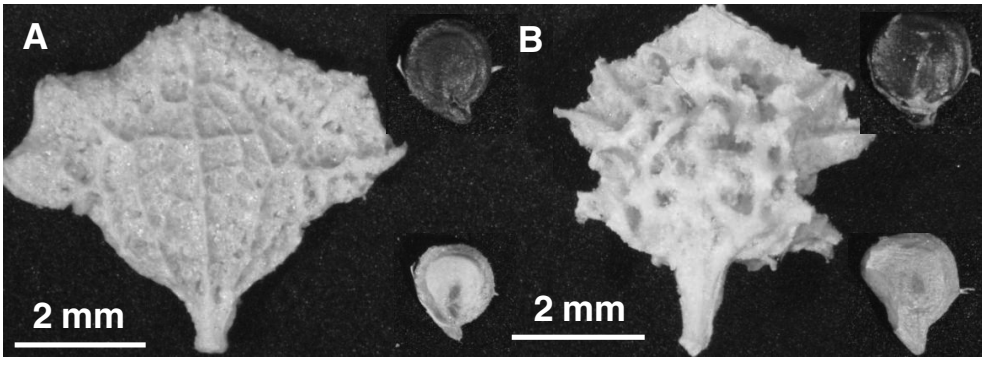

Figure 1. *Atriplex centralasiatica* (A) Flat fruit and their brown and black seeds. (B) Humped fruit and their brown and black seeds.

Effect of cold stratification on germination

A two-way ANOVA of final germination percentage showed significant ($P < 0.01$) effects of seed colour. Cold stratification did not affect final germination percentage of either seed (figure 2). However, the rate of germination increased after cold stratification, with an increase in the germination index of brown seeds in 360 mM NaCl treatment from 22.0 to 42.9; and of black seed in 240 mM NaCl treatment from 19.6 to 31.7.

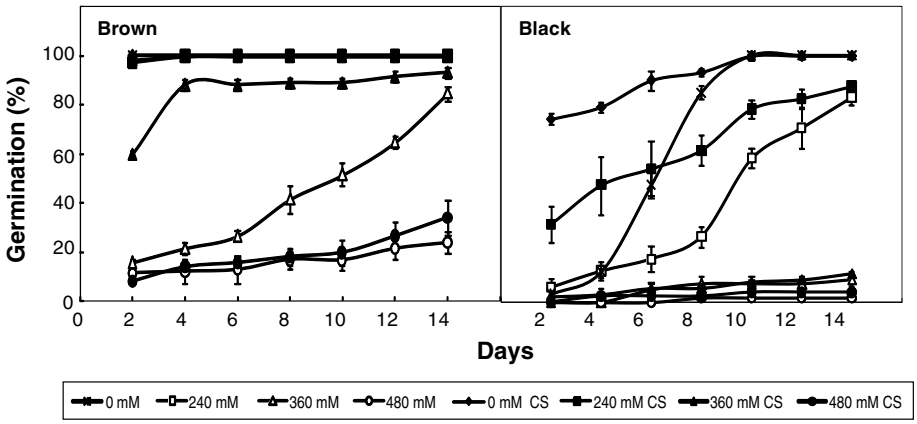

Figure 2. Germination percentage of non-treated and 4 day cold-stratified (CS) brown and black seeds of *A. centralasiatica* in 0, 240, 360 and 480mM NaCl solution at 20/30°C temperature regime. To calculate the average rate of germination more than 100 seeds were used for each treatment. The experiments were repeated 3 times and S.E was calculated.

Effect of scarification on germination

Scarification significantly increased final germination percentage (figure 3). Germination index was also significantly increased for brown seeds in 360 mM NaCl treatment from 22.0 to 49.8, and for black seeds in 360 mM NaCl treatment from 2.6 to 20.2.

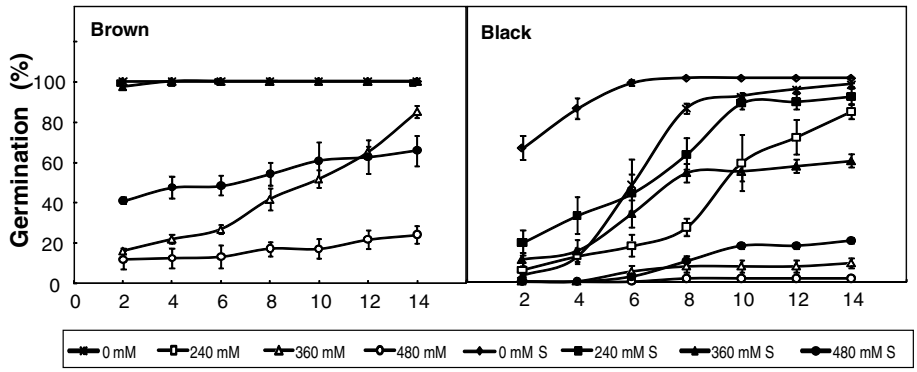

Figure 3. Germination percentage of non-treated and scarification (S) of brown and black seeds of *A. centralasiatica* in 0, 240, 360 and 480mM NaCl solution at 20/30°C temperature regime. To calculate the average rate of germination more than 100 seeds were used for each treatment. The experiments were repeated 3 times and S.E was calculated.

Effect of plant hormones on germination

Generally, 1 mM ACC, 10 μM BA and 10 μM GA_4 did not promote the germination of black and brown seed under conditions of salt stress (figure 4). ABA at 10 μM and 50 μM PAC showed little effect on germination of black and brown seeds in distilled water (Data not shown). However, in the presence of salt stress (240 or 360 mM), 10 μM ABA significantly inhibited germination of black and brown seeds; black seeds were more sensitive to ABA than brown seeds under salinity stress (figure 4). PAC at 50 μM significantly inhibited germination of black seeds, but showed little effect on germination of brown seeds (figure 4). Flu treatments improved the germination of both black and brown seeds under salinity stress (figure 4).

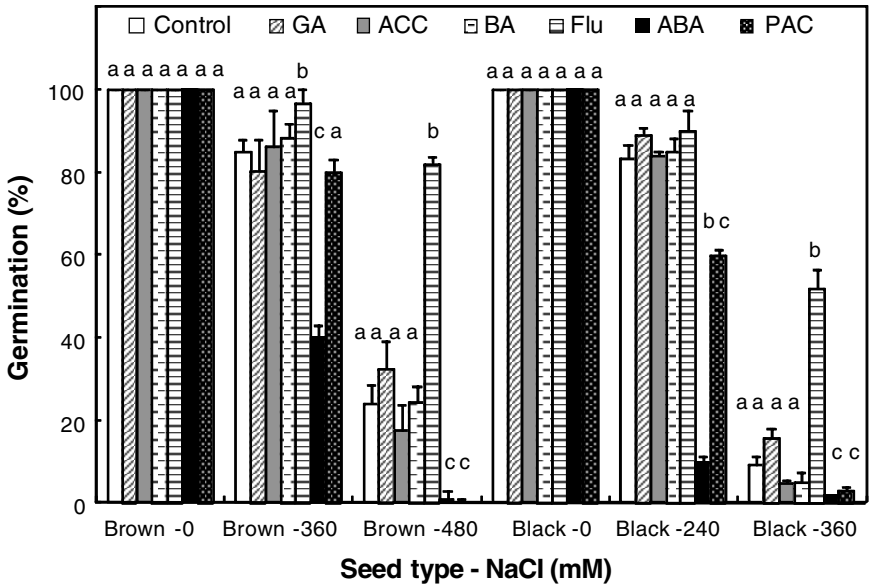

Figure 4. Germination percentage of non-treated (control), GA_4 (10μM), ABA (10μM), BA (10μM), PAC (50μM), Flu (10μM) and ACC (1mM) of brown (Br) and black (Bl) seeds of *A. centralasiatica* in 0, 240, 360 and 480mM NaCl solution at 20/30°C temperature regime. To calculate the average rate of germination more than 100 seeds were used for each treatment. The experiments were repeated 3 times and S.E was calculated.

Effect of stratification, scarification and salt on endogenous ABA and GAs content in brown and black seeds of A. centralasiatica.

Dry brown seeds contained more active forms of GAs than dry black seeds (table 1). However, the contents of endogenous ABA for brown and black seeds were similar (table 1), while the ABA content in humped bracteoles (140 ± 8.36 ng g^{-1}) was higher than flat bracteoles (80.5 ± 4.6 ng g^{-1}).

After imbibition in water or salt solution for one day, GA_1 and ABA content decreased and GA_4 content increased comparing to dry seeds. Cold stratification, coat scarification and salt treatments showed lower GAs than the control in brown seeds; however, the content of GA_1 was slightly higher in black seeds than in the control.

Table 1. Content of ABA and Gibberellins in dimorphic seeds of *A. centralasiatica* after stratification, scarification and salt (360 mM NaCl) treatments (Average ± S.E. ng g^{-1}).

	GA_1		GA_4		ABA	
	Brown seeds	Black seeds	Brown seeds	Black seeds	Brown seeds	Black seeds
Dry seeds	7.80±0.50	0.10±0.00	0.23±0.03	Not detected	60.80±2.30	62.90±4.60
Control	3.90±0.09	0.04±0.01	1.24±0.11	0.02±0.01	14.23±1.24	19.23±1.04
Cold Stratification	3.57±0.07	0.06±0.01	0.46±0.04	0.02±0.01	11.56±3.45	14.46±1.23
Coat scarification	2.08±0.39	0.08±0.01	0.40±0.19	0.03±0.01	13.87±3.76	18.43±2.24
Salt	3.78±0.76	Not detected	0.34±0.45	Not detected	22.78±3.55	24.76±2.33

The ABA content after cold stratification treatment was lower than the control in both black and brown seeds, while ABA content after coat scarification treatment was similar to control in both seeds. However, the ABA content after salt treatment was higher than control in brown seeds but similar in black seeds.

Discussion

The experiment not only showed differences in germination, but also in the size of seedlings from black and brown seeds (figure 1). Seedlings from brown seeds were bigger than seedlings from black seeds. One reason might be that brown seeds were bigger than black seeds, and contained bigger embryos (Li *et al*., 2008a). Another reason might be that brown seeds contained more GAs than black seeds (table 1); or brown seeds were more efficient than black seeds in degrading the ABA, although they contained similar levels of ABA in dry seeds (table 1). Recent investigation on *Suaeda splendens* also showed differences in the salt tolerance in terms of growth of seedlings from black and brown seeds (Redondo-Gómez *et al*., 2008).

North China has a monsoon type climate and the rainfall is highly variable; mean annual precipitation is 500-600 mm, 80% of which occurs from June to September (Jin *et al*., 1999). In saline areas, surface fresh water is scarce but shallow saline groundwater is widespread. As a result, occurrence of spring drought and autumn flood are common in the saline area. Under such unpredictable and stressful environment, the production of a number of morphological and physiological adaptations in seeds that affect their dormancy and germination shows an adaptation advantage. Brown seeds are tolerant to the high salinity and drought (Li *et al*., 2008a) that is common in spring and may therefore germinate and grow in spring. Black seeds are sensitive to salinity and may therefore not germinate in spring but in summer when rainy season sets in and soil starts getting desalinized. Black seeds' dormancy in salt stress (figure 2, 3) can help in synchronizing the germination with the occurrence of appropriate environmental conditions so that the chances of seedling survival are increased. Similar results have been observed in case of

other dimorphic seeds (Harper, 1977; Baskin and Baskin, 1998; Li *et al*., 2008b). The production of brown and black seeds as well as different seedlings might be therefore a kind of bet-hedging strategy (Imbert, 2002; Li *et al*., 2008a) for *A. centralasiatica* adapted to saline soils and monsoon climate.

We propose that black seeds may be the main part of the seed bank under the condition of north China, for several reasons: 1) The black seed coat restricts water absorption (Li *et al*., 2008a), enhances tolerance to mechanical damage and fungal infection, and delays germination; coat scarification increased germination percentage (figure 3). 2) Black seeds have dormancy in salt stress (figure 2, 3) and they keep dormancy and vigour for a longer time after leaving mother plant than brown seeds. 3) Black seeds are sensitive to light, salinity, temperature and their interaction (Li *et al*., 2008a) and remain dormant in saline soils. Similar results have been reported in *Atriplex sagittata* (Mandák and Holmanová, 2004), rape seed (Zhang *et al*., 2006), and *S. salsa* and *S. aralocaspica* (Li *et al*., 2008b; Wang *et al*., 2008).

There have been few studies on the dynamic changes in germination response to salinity when dormancy conditions of halophyte seeds were changed (Want *et al*., 2008). Our results showed that after 4 days cold stratification, germination velocity of black and brown seeds in salinity were significantly higher than those that had not been stratified. Therefore, cold stratification increases growth potential of embryo so that the radicle can break through the seed coat resulting in germination. Similar results were shown in some *Suaeda* species (Li *et al*., 2008b; Wang *et al*., 2008). The main mechanism might be the degradation of endogenous ABA (table 1).

The fact that seed coat scarification alleviated salinity stress on germination would imply that embryo growth potential was low in the presence of salinity stress. Scarification decreased the mechanical resistance of the seed coat to the embryo to the point at which the radicle could elongate, and thus the seed could germinate. Scarification showed little effect on content of endogenous ABA and a reduced content of GAs (table 1), implying that decreasing mechanical resistance is the main mechanism in promoting seed germination. However, in *Suaeda physophora* and *Haloxylon ammodendron*, seed coat scarification did not affect seed germination under salinity stress (Song *et al*., 2007). This suggested another possible mechanism which affects seed germination via the seed coat, such as inhibiting sodium influx into and potassium efflux from the embryo to protect the seed from ion toxicity (Song *et al*., 2007).

Halophytes germinating under saline conditions face osmotic stress and ionic toxicity, which decreases strength of the radicle to push through the outer covering. In addition, salinity stress stimulates the production of ABA which inhibits seed germination, ethylene perhaps effectively countering both ABA and low vigour effects and promotes seed germination. An inhibitor of ABA biosynthesis (Flu) promoted seed germination in salinity stress (figure 4) which implied that ABA was involved in the germination process in salinity stress. This was also confirmed by exogenously applied ABA (figure 4) and the content of endogenous ABA (table 1). ABA content in bracteoles was higher than that of seeds, which implied that ABA in bracteoles might inhibit seed germination, but we cannot exclude the presence of other germination inhibitors in the bracteoles, such as some allelopathic compounds or salts.

Exogenous GA_4 could not alleviate seed germination of *A.centralasiatica* in salinity (figure 4). Gibberellins are reported to have differential response in the germination of halophytes. Seed germination under saline conditions was almost completely alleviated in *A. stocksii* and *S. salsa* (Khan and Rizvi, 1994; Li *et al*., 2005), while species like *Triglochin maritima, Sporobolus ioclados, Urochondra setulosa, Suaeda fruticosa, Salsola imbricata* and *Haloxylon stocksii* failed to respond to any gibberellic acid treatment (Khan and Ungar, 2000). PAC inhibited black seed germination of *A. centralasiatica* in salinity, but showed little effect on brown seeds (figure 4). One reason might be that brown seeds contain higher active GAs than black seeds (table 1).

Contents of active GAs (GA_1 and GA_4) in brown seeds were about 20-80 times higher in comparison to black seeds both in dry and those imbibed in salt or subjected to cold stratification and/or coat scarification treatments. ABA contents of black and brown seeds are, however, similar (1.03-1.35 times). It appears that the role of GA is important in determining the difference between the germination of black and brown seeds in salt, PAC and ABA treatments (figure 4). Substrate salinity inhibited seed germination perhaps through decreasing concentration of GA and increasing content of ABA. Brown seeds might synthesize GA more than black seeds when they were growing in the mother plant, which directly or indirectly affected seed germination through changing the content of ABA which ultimately regulates seed dormancy in later stage (Kucera *et al*., 2005).

In conclusion, the production of black and brown seeds of *A. centralasiatica* showed bet-hedging ecological strategies. GAs in seeds and ABA in seeds and bracteoles were the main plant hormones that affected the difference of black and brown seeds germination in salinity stress.

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