The Asian and Australasian Journal of Plant Science and Biotechnology ©2010 Global Science Books

Characterization and Cryopreservation of *Malus sieversii* Seeds

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ABSTRACT

Kazakhstan is one of the centers of origin and evolution of apple (*Malus*). The main forest species is *Malus sieversii* (Ledeb.) M. Roem., representatives of which have important traits such as disease and insect resistance, cold hardiness, and fruit quality. The area of these unique wild forests is continually decreasing as a result of human activities. An expedition was held in September 2008 to collect and safeguard the genetic resources of *Malus sieversii* in Kazakhstan. Over 4400 seeds from the fruit of 34 *Malus sieversii* accessions were collected in the Zailyiski Alatau Mountains near Almaty (Bel-Bulak and Issyk Gorges). The trees where fruit was collected showed high variability in habit and fruit size, shape and flavor. Fruit color varied from yellow white to red. Seeds were variable in both color and length. We tested seed cryopreservation for long-term storage of wild *Malus* biodiversity. Seeds were air dried to a mean moisture content of 8.23%. Seeds were directly immersed in liquid nitrogen (LN) for 30 min and rewarmed at room temperature to test the effect of LN exposure. Viability following LN as evaluated by 2,3,5-triphenyltetrazolium chloride (TZ) ranged from 72.4% to 90.0% and did not differ significantly from the controls or from the germination tests. Germination tests indicated slightly but not significantly higher viability for LN exposed seeds, but germination vigor for LN exposed seeds was significantly higher than controls over the 6 week germination period. These results indicate that cryopreservation in LN is an option for long-term conservation of this important germplasm.

Keywords: germplasm, liquid nitrogen, long-term storage, wild apple
Abbreviations: LN, liquid nitrogen; **TZ**, 2,3,5-triphenyltetrazolium chloride

INTRODUCTION

Kazakhstan is widely known as center of origin for apple, one of the most important temperate fruits (Vavilov 1930). Vavilov noted the large diversity of wild-apple trees available through the gorges of the Tyan-Shan Mountains near Almaty with apples that range from typical wild-tart-inedible small fruit to forms with large sweet fruits (Vavilov 1987). Some of the wild-apple trees have fruit that is suitable for production without additional breeding. Vavilov proposed that the regions with the greatest diversity are likely the area of the species origin.

The Republic of Kazakhstan has three species of wild apple, but the predominant species is *Malus sieversii* (Ledeb.) M. Roem. Decades of study of the wild apples in the mountain systems of Kazakhstan conducted by A.D. Djangaliev revealed high polymorphism of *M. sieversii* for size, shape, colour and flavor of fruits, time of maturity and many other valuable features (Djangaliev 1987; Djangaliev *et al*. 2001).

Field estimation of the samples collected during several joint Kazakh-American expeditions revealed similarity in fruit quality between some forms of *M. sieversii* and cultivars of *M. domestica* (Forsline 1995; Forsline *et al*. 1994). Molecular markers for comparative analysis of DNA revealed close similarity between Kazakhstan apples and cultivated apples (Robinson *et al*. 2001; Harris *et al*. 2002). Thus the hypothesis that the wild apple of Kazakhstan is the ancestor of most modern cultivars of this important culture is confirmed. Unfortunately the area of these global valuable unique wild forests is highly decreasing as a result of human activities and climate change.

Cryopreservation of germplasm in liquid nitrogen is becoming widely used as an additional tool for conservation of biological diversity (Engelmann 2004; Reed 2008). Cryogenic storage at ultra low temperature (-196°C) as a backup to *in situ* and *ex situ* collections reliably safeguards these collections from unfavorable environmental factors, dangerous diseases and insects. Cryopreserved plant cells, tissues and organs retain viability, regeneration potential and genetic stability for long periods and allow the regeneration of the whole plant as needed. Cryopreservation of orthodox (desiccation tolerant) seed provides long-term storage for many species and most orthodox seed can be stored if the moisture content is reduced to between 5 and 10% (Stanwood 1985). Recent papers showed high viability of fruit-tree seeds after liquid nitrogen immersion for pear (Reed *et al*. 2001) and wild-apple species (Safina 2008).

The strategy we developed for cryopreservation of Kazakhstan's apple germplasm takes into account the requirements of both the vegetative propagated cultivars and the wild species. For cultivars, hybrids and breeders selections that must be vegetatively propagated, we developed a protocol for cryopreservation of shoot tips isolated from *in vitro* plantlets (Kushnarenko *et al*. 2009). This protocol results in 60-80% shoot tip recovery for cultivars (*Malus domestica* Borkh.) and selections (*Malus sieversii* (Ledeb.) M. Roem). For conservation of wild-apple diversity the cryopreservation of the orthodox seeds appears to be the best method for efficient storage. The objective of this study was to study the diversity of *Malus sieversii* (Ledeb.) M. Roem in the Zailyiski Alatau Mountains near Almaty and determine the effect of liquid nitrogen exposure on freshly collected seeds of the diverse accessions.

MATERIALS AND METHODS

Germplasm collection

Fruit of 34 *Malus sieversii* accessions were collected during an

Received: 11 June, 2009. Accepted: 23 November, 2009.

Original Research Paper

expedition in September 2008 in the Zailyiski Alatau Mountains near Almaty (Bel-Bulak and Issyk gorges). Each accession was documented with the following data: locality of collection including GPS latitude, longitude and elevation (eTrex, Garmin); description including height and habit of the tree, size, shape, color and flavor of fruit. The seeds were extracted from the fruit, allowed to air dry and collected in paper packets.

Seed moisture content

For estimation of moisture content, ten replicates of 10 seeds each were placed in glass beakers with sintered glass stoppers and dried in an oven for 16 h at 103°C. The seeds were allowed to cool above silica gel in a desiccator. Moisture content was calculated as follows: (% Seed moisture content = [(Fresh weight – Dry weight) /Fresh weight] X 100).

Liquid nitrogen exposure

Air-dried seeds were placed in plastic 1.2 ml cryovials (10 seeds per cryovial), attached to aluminum canes and directly immersed in liquid nitrogen. After 30 min immersion in LN seeds were rewarmed for 30 min at room temperature. Viability of the seeds was evaluated using germination and TZ tests. Six replicates (3 for TZ tests and 3 for germination tests) of 10 seeds each were used for each of 5 seed accessions. Sixty seeds of each sample were held at room temperature as a control.

Tetrazolium testing and seed germination

For TZ tests, the seeds were soaked in warm tap water (25°C) for 3 to 4 h, and stored between damp paper towels overnight to soften the seed coats. Seeds coats were removed with forceps and scalpel and the embryos placed in a 1% TZ solution for 3 to 4 h (DiMaio and Shilito 1989). Seeds were evaluated using a viability scale of TZ-stained seeds developed for pear (Reed *et al*. 2001). Pink staining of embryos indicated viable seeds. Seeds with radicals or epicotyls that remained white were considered non viable.

For germination tests, plastic 100 ml beakers were filled with Perlite (2/3 of the volume of beaker) soaked with tap water. Seeds were surface sterilized for 10 min in a 1% "Deochlor" solution, rinsed with tap water and planted 1 cm deep in the Perlite, 10 seeds per beaker and 3 beakers per genotype. The beakers were covered with plastic lids and placed in the cold room (4°C) for 8 weeks to stratify the seeds. After 8 weeks the lids were removed and the beakers transferred to a plant growth room at 24°C with a 16 h light/8 h dark photoperiod (light intensity of 25 $\mu mol \cdot м^{-2} \cdot s^{-1}$). Germination and plantlet production were determined after 5 weeks in the growth room. Germination was defined as the emergence of the radical and plantlet production as the growth of primary leaves. Vigor was determined by the mean time required for emergence of the seedlings.

Data analysis

Data for seed germination was taken weekly for 6 weeks after seeds were moved from the cold room to the growth room. Vigor data was taken weekly for 6 weeks. Mean seedling emergence and TZ viability data were compared by *t*-tests ($P \leq 0.05$) using SYSTAT software. Data are presented as means and standard deviations. Seed sizes of 10 seeds were measured for five accessions. Lengths and widths were compared with paired *t*-tests ($P \leq 0.001$) using SYSTAT12 software.

Table 1 Description of *Malus sieversii* accessions collected from the Zailyiski Alatau Mountains near Almaty (Bel-Bulak gorge, 43°, 15'N; 77°10'E).

Accession	Elevation (m)	Tree descriptions			Fruit description			
		Habit	Height (m)	Age (years)	Size	Shape	Color	Taste
MS1	1320	spreading round	5-6	15	medium 6 × 6 cm	round-conic	light yellow	sweet-sour pleasant
MS2	1370	drooping	10	30-40	small 4 × 5 cm	round	light yellow	sour
MS3	1416	spreading oval	6	40-50	medium	round	yellow red blush	sweet-sour
MS4	1332	spreading	5	25	small 4 × 4.5 cm	round	yellow red strips	sweet-sour pleasant
MS5	1300	spreading round	7-8	35-40	medium	conic	light green red blush	fresh sweet
MS6	1310	drooping round	9	50-60	medium 6 × 6 cm	round	yellow red blush	sweet-sour
MS7	1310	raised round	8	50-60	small 3 × 4 cm	cask shaped	yellow	acidic
MS8	1280	spreading round	6	50-55	medium 5 × 4.5 cm	oval	yellow red blush	sweet-sour
MS9	1220	spreading round	7	50-60	large 7 × 7 cm	round	yellow	sweet-sour
MS10	1210	spreading broom shaped	10	50-60	medium 5 × 6 cm	conic	yellow red blush	sweet-sour
MS11	1220	spreading round	8-9	50-60	medium 4.5 × 5 cm	oval	yellow	sweet-sour tart
MS12	1160	drooping oval	5-6	30-40	small 3.5 × 3.5 cm	conic	yellow	fresh sweet
MS25	1340	spreading ball shaped	5	35-40	small	conic	light yellow	fresh sweet
MS26	1330	spreading	8	40-50	medium	round conic	light yellow	sweet-sour pleasant
MS28	1310	spreading	16-18	100	large	conic	yellow red stripes	sweet-sour pleasant
MS31	1280	spreading	14	30-40	small 3.5 × 4 cm	round	yellow	sweet-sour
MS32	1240	round	12	25-30	medium	round flat	light yellow	sweet-sour
MS33	1160	spreading	16-18	60	large	round	light yellow	sweet-sour
MS34	1100	drooping	15	40	small 4 × 4 cm	round	yellow red stripes	fresh sweet

Table 2 Description of some *Malus sieversii* accessions collected from the Zailyiski Alatau Mountains near Almaty (Issyk gorge, 43°, 15'N; 77°30'E).

Accession	Elevation (m)	Tree descriptions			Fruit descriptions			
		Habit	Height (m)	Age (years)	Size	Shape	Color	Taste
MS13	1740	spreading round	4	30-35	medium 4 × 5 cm	round ribbed	green red blush	sweet-sour
MS14	1710	round	4	40-50	small 3 × 4 cm	round ribbed	dark red	sweet-sour
MS15	1705	oval	5	60-70	medium 4 × 5 cm	cask-shaped, ribbed	green red blush	sweet-sour
MS16	1705	oval	4	30	small 3.5 × 3.5 cm	round	yellow	fresh
MS17	1680	spreading round	3.5	30	small 3 × 3 cm	round	yellow	sweet-sour bitter
MS18	1680	round	3.5-4	20	medium 4 × 5.5 cm	cask-shaped	yellow pink blush	sweet-sour
MS19	1650	spreading round	6	50-60	small 2.5 × 2.5 cm	round	yellow green	sweet-sour bitter
MS20	1640	spreading round	5.5-6	40-50	large 5 × 6 cm	round-conic	yellow	sweet-sour
MS21	1600	oval	4.5-5	15-20	medium 4 × 5 cm	round-conic	green red blush	sweet-sour
MS22	1480	round	6	15-20	small 2.5 × 2.5 cm	round	white red blush	sweet-sour
MS23	1400	oval	5	30-40	large 5 × 6 cm	round	yellow	sweet-sour bitter
MS24	1300	oval	3.5	15	medium 3 × 5 cm	cylinder shaped	green red blush	sweet-sour tart

RESULTS AND DISCUSSION

The plant collection expedition collected over 4400 seeds from 34 accessions of *M. sieversii*. Twenty two accessions (No. 1-12; 25-34) were collected in the Bel-Bulak gorge (43° 15' N; 77° 10' E; elevation from 1160 to 1370 m above sea level); twelve accessions (No. 13-24) – in the Issyk gorge (43° 15' N; 77° 30' E; elevation from 1300 to 1740 m above sea level). It should be noted that in 2008 the wild apple crop was sparse due to a severe spring frost. This may have resulted in selection of frost tolerance or late blooming genotypes.

The fruit collected showed high variability in habit and fruit size, shape and taste (**Tables 1, 2**). Trees varied in growth habit with spreading, oval, round and broom shaped and from 3.5 m to 18 m in height. Tree age ranged from 15 years to 100 years with most in the 40-60 year range. Collections were made from trees growing from 1100 m to 1705 m in the two canyons. Productivity varied but some accessions were highly productive (**Fig. 1A**). Fruit color varied from green to light yellow or yellow with a blush to red striped or dark red (**Fig. 1B-D**) and flavors were described as acidic, bitter, sweet, sweet-sour and fresh. Fruit size and shape ranged from small to large and round to cylindrical to conic, ribbed or flat (**Tables 1, 2**). The color of the seeds varied from light to dark brown and the shape also varied (**Fig. 2**). There were significant ($P \leq 0.001$) differences in the mean lengths of the seed accessions collected but the mean widths were not significantly different (**Fig. 3**). These data agree with the estimation of *M. sieversii* by Djangaliev (Djangaliev 1987; Djangaliev *et al*. 2001) as a highly polymorphic species.

Fig. 1 ***Malus sieversii*** **(Ledeb.) M. Roem. accessions.** (**A**) No. 12; (**B**) No. 30; (**C**) No. 27; (**D**) No. 24 collected from the Zailyiski Alatau Mountains near Almaty.

Fig. 2 Seeds of ***Malus sieversii*** **(Ledeb.) M. Roem. accessions.** (**A**) No. 22; (**B**) No. 9; (**C**) No. 13; (**D**) No. 4 collected from the Zailyiski Alatau Mountains near Almaty. Note variability in testa color.

Air dry seed moisture content of *M. sieversii* varied from 7.8 to 8.6% with a mean value of 8.23 ± 0.24%. For most orthodox (desiccant-tolerant) seeds (like apple seeds), the optimum moisture content for seed cryopreservation ranges from 5 to 10% (Stanwood 1985). In recent papers several cryopreservation regimes for apple and pear seeds were compared (Reed 2001; Safina 2008). For pear seed cryopreservation four treatments were tested (Reed *et al.*

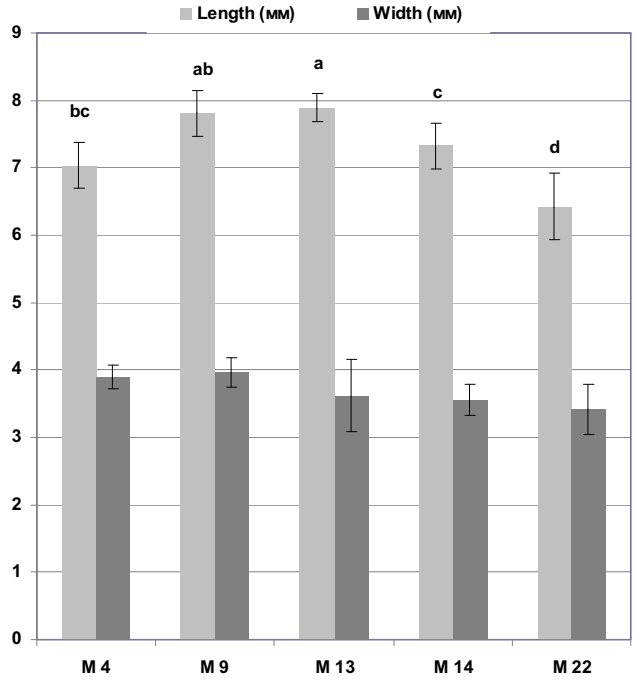

Fig 3 Mean seed length and width of five *Malus sieversii* seed accessions (n=10). Means followed by different letters are significantly different ($P \leq 0.001$).

Fig. 4 Staining of embryos of *Malus sieversii* accession (No. 4, non frozen control) with 2,3,5-triphenyltetrazolium chloride (TZ). Pink staining of 6 embryos (left side) indicates viable seeds; white staining of 3 embryos (right side) indicates non viable seeds.

Table 3 Mean % viability and germination of *Malus sieversii* seeds as determined by triphenyl tetrazolium chloride (TZ) and after 5 weeks of germination.

Accession	Frozen in LN		Control not frozen	
No	TZ viability test	Germination test	TZ viability test	Germination test
MS4	72.2 ± 6.9 a	80.0 ± 0 a	70.6 ± 4.2 a	66.7 ± 5.8 a
MS9	73.3 ± 5.8 a	80.0 ± 10.0 a	76.7 ± 11.6 a	73.3 ± 5.8 a
MS13	83.0 ± 5.1 ab	76.7 ± 5.8 a	90.0 ± 10.0 a	66.7 ± 11.5 a
MS14	81.5 ± 6.4 a	90.0 ± 10.0 a	80.0 ± 10.0 a	83.3 ± 15.3 a
MS22	75.9 ± 5.3 ab	86.7 ± 5.8 a	83.3 ± 5.8 a	73.3 ± 11.5 a

n = 30; Mean ± standard deviation. Means in a column with the same letter are not significantly different ($P \leq 0.05$). T test indicated no significant differences in rows.

2001). Exposure by direct immersion and removal or direct immersion and 1 min vapour phase at removal or 2 minutes vapour phase before immersion and direct removal or 1 min in vapour phase removal were not significantly different for pears tested with TZ, but direct immersion and removal resulted in significantly better greenhouse germination than the other treatments (Reed *et al.* 2001). Eight *Malus* species seeds were tested for cryopreservation (*M. baccata* var *sibirica*, *M. purpurea*, *M. floribunda*, *M. sargentii*, *M. soulardii*, *M. cerasifera*, *M. orientalis*, *M. domestica*) and viability ranged from 69.2 to 100% after LN (Safina 2008). This is similar to the 72.4 to 90.0% viability we found with *M. sieversii* (**Table 3**). Seed cryopreservation of 54 species from 23 families of the flora of Far East of Russia demonstrated that storage in LN doesn't decrease germination for 80% of the species tested, and resulted in significantly improved germination for 13% of the species (Voronkova *et al.* 2003).

Since earlier studies found that direct immersion in LN and rewarming at room temperature was the most effective, that technique was used for testing the *M. sieversii* seed accessions. TZ tests clearly indicated live and dead seeds (**Fig. 4**). The five accessions tested varied in viability after LN exposure according to the TZ tests, but the control data were not variable (**Table 3**). Germination tests for control and LN exposed seed indicated no significant differences among the accessions. In addition the TZ tests and germination tests of control and LN exposed seeds of each accession were not significantly different. This indicates that either test can be used to determine the viability of LN-stored apple seed.

Exposure to liquid nitrogen had a stimulatory effect on the rate of seed germination for both *M. sieversii* accessions tested (**Fig. 5**). Seedling emergence was significantly higher ($P \leq 0.001$) at all time points for LN exposed seeds compared to control seeds. The first LN-exposed seedlings emerged 1 week after transfer to the growth room, while the controls required 2 to 3 weeks for first emergence. Liquid

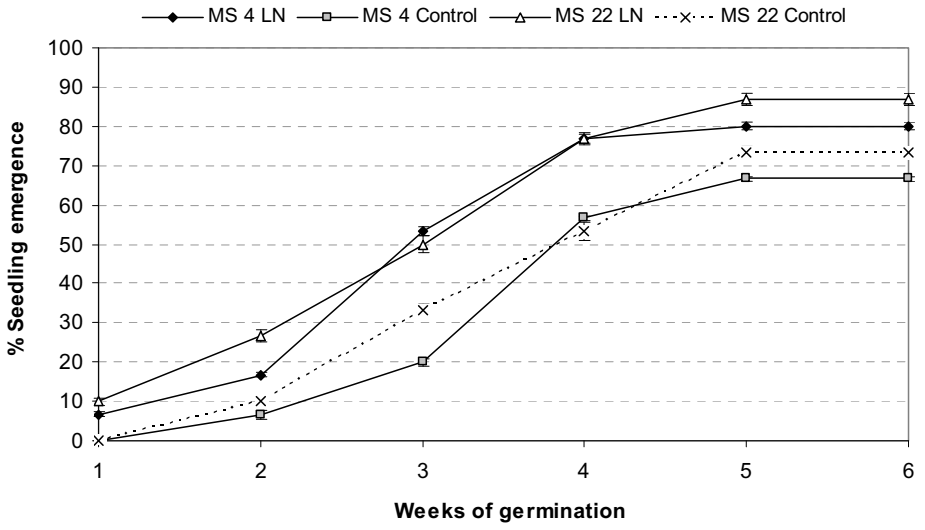

Fig. 5 Seedling emergence for two *Malus sieversii* seed accessions over a 6 week period. LN seeds were placed in liquid nitrogen for 30 minutes before stratification, control seeds remained at room temperature. All seeds were stratified for 8 weeks before germination. (n=30).

nitrogen stimulation of seed germination has been noted in other papers (Reed 2001; Voronkova *et al*. 2003) and is possibly a result of scarification of the seeds or release from dormancy induced by ultra low temperature.

This study documents the collection of diverse *M. sieversii* germplasm and storage of the seed in liquid nitrogen. The diversity of the collection was confirmed by analysis of the tree morphology data and the seed characteristics. Our results confirm that air-dried seeds of *Malus sieversii* are similar in LN response to other orthodox seeds and that this valuable germplasm can be safely stored in LN. As a result of this study the 34 seed accessions of *M. sieversii* germplasm are stored in LN as a secure backup for the diverse *ex situ* plants in the Zailyiski Alatau Mountains of Kazakhstan.

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