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Ex situ genetic conservation potential of seeds of two high elevation white pines

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Abstract Genetic variation in a plant species is a key to its ability to survive and evolve in the face of changing environmental pressures. Due to insect and disease impacts, changes in fire regimes, and a changing climate, many populations of high elevation white pine species continue to experience high mortality levels and potentially worrisome decreases in genetic variation. In recent years, some trees rated highly for resistance to the non-native white pine blister rust have been killed by fire or mountain pine beetle. Ex situ genetic conservation offers the possibility to conserve the genetic variation within a species before much of it is lost. For many conifer species, freezer storage of seed offers a relatively inexpensive, long-term method of storing germplasm for future use. However, there is uncertainty concerning how long seed of some conifers can be stored and retain viability. We report here on results of germination testing of the oldest known seedlots of whitebark pine (*Pinus albicaulis* Engelm.) and foxtail pine (*P. balfouriana* Grev. & Balf.), some of which had been in storage for several decades. The 52 whitebark pine seedlots averaged 47.7% germination (average seed age of 19.2 years), while the four foxtail pine seedlots had an average germination of 71.3% (average seed age of 15.3 years). Some seedlots of both species had greater than 90% germination. Refinements to the stratification procedure have since been developed which should enhance germination further. A follow-up study examining seedling vigor of long-stored whitebark pine seed is planned.

Keywords Genetic conservation · Ex situ · Whitebark pine · Foxtail pine · Seed storage

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Introduction

Whitebark pine (*Pinus albicaulis* Engelm.) and foxtail pine (*P. balfouriana* Grev. & Balf.) are two of the eight species of white pines (or five-needle pines) native to forest ecosystems of the western U.S. and Canada. Both these species are part of the subgenus *Strobus* within the genus *Pinus* but are placed in two different sections within the subgenus, *Quinquefoliae* for whitebark pine and *Parrya* for foxtail pine (Gernandt et al. 2005). These species are facing threats from numerous factors, including a change in fire regimes, climate change, insects, and high susceptibility to the disease white pine blister rust (WPBR), caused by the non-native fungal pathogen *Cronartium ribicola* J.C. Fisch. in Rabh. (Tomback and Achuff 2010). In the U.S. whitebark pine has been proposed for listing under the Endangered Species Act (U.S. Fish and Wildlife Service 2011), and in Canada it has been designated as an endangered species under the Species at Risk Act (Government of Canada 2012). Foxtail pine, endemic to northern California, is listed as 'near threatened' on the IUCN Red List (Farjon 2013). To varying extents, programs are underway for all eight species to evaluate the level and geographic distribution of genetic resistance to WPBR (Sniezko et al. 2011a, 2014, 2016). Extensive screening results of whitebark pine indicate that although most trees are very susceptible, a wide range of variation in genetic resistance to blister rust exists within the species (Sniezko et al. 2007, 2011a). Early results from a current ongoing inoculation trial of foxtail pine seedlings indicate that this species is very susceptible to WPBR (Sniezko unpublished data).

Concerns about the high mortality in some of the white pine species and the vulnerability of many of their populations have led to the initiation of genetic conservation efforts, including ex situ conservation via freezer storage of seed (Sniezko et al. 2011b). Freezer storage of seed is widely used as a long-term means of preserving the genetic variation in plants for possible future need (Fowler 2016; Broadhurst et al. 2016; Kolotelo et al. 2001; Ledig 1986, 1988; Alberta Sustainable Resource Development 2009). As part of the large recent genetic conservation effort in the U.S., over 1000 individual tree seedlots of whitebark pine and the other high elevation white pine species have been collected since 2008 and are stored at the National Laboratory for Genetic Resources Preservation (NCGRP) in Fort Collins, Colorado and recorded in the GRIN-Global database (2016). Additional collections and duplicates of many collections at NCGRP are also stored at United States Department of Agriculture (USDA) Forest Service regional facilities. However, for many species, including the white pines, uncertainty still remains concerning how long seed can remain in freezer storage and retain its viability, knowledge that is vital for success of the current genetic conservation strategy of tree species.

Of the nine species of white pines native to the United States or Canada, the most uncertainty concerning long-term seed storage involves whitebark pine. There is relatively little information about seed viability after prolonged storage for this species. Losses in germination of 30–65% have been reported after one or 2 years of freezer storage (Burr et al. 2001), and even higher losses, 97–99%, have been reported for seed after 11 years of storage (McCaughey and Schmidt 1990). Earlier reports also noted a decline in germination over 20 years from 50% viability to 3% (Schubert 1954), and from 24 to 1% after 11 years (Mirov 1946). However, it has also been reported that some whitebark pine seedlots showed relatively little loss in germination after 10 years of storage (Berdeen et al. 2007; Bower et al. 2011). Current guidelines for 'safe times to cold- and freeze-store dry, mature seeds' (and achieve germination of at least 50%) are 8 years for whitebark pine and 17–18 years for foxtail pine (Krugman and Jenkinson 2008). However, these

guidelines are based on a relatively small amount of early work with these species reported between 1946 and 1969 and summarized by Krugman and Jenkinson (1974).

More information is needed to assess the viability of whitebark pine and other white pine species seed that have undergone long-term storage. In this study, undertaken at Dorena Genetic Resource Center (DGRC), Cottage Grove, Oregon, we examine germination of the oldest available seedlots to date for whitebark pine and foxtail pine. The results of this study provide guidance for the ex situ genetic conservation efforts currently underway.

Materials and methods

Plant material

The 57 seedlots included in this study were selected based on age and availability; the oldest seedlots had been in storage for more than 30 years. The 53 whitebark pine seedlots spanned a range of cone collection years (1978–2009), were contributed by various U.S. and Canadian organizations, and covered much of the geographic range of whitebark pine (Table 1). All seeds were collected from natural stands, and each seedlot was from a single parent tree, except for 10 bulked seedlots of whitebark pine and one of foxtail pine. In some cases multiple seedlots from a contributing facility were from a single collection year (2–20 seedlots), while in other cases they were from multiple collection years. A single bulked seedlot collected in 1996 on the Shoshone National Forest, stored by two separate USDA Forest Service (USFS) groups since around 2001, was also included (Table 1). A 1-year-old seedlot (collected in 2009) of whitebark pine from Oregon was included as a control.

Three of the four foxtail pine seedlots for this study were from 1994 collections in northern California, two from the Klamath National Forest and one from the Shasta-Trinity National Forest, and were provided by the Pacific Southwest Region (Region 5) of the USDA Forest Service. A fourth seedlot, also from California, but of unknown provenance, was collected in 1997 and provided by a seed dealer to the Rocky Mountain Research Station (RMRS) where it had been stored in a chest freezer since 2002.

Most of the seedlots had been in freezer storage (approximately −16 °C) and at moisture content of <10%, which is within the range generally recommended for most conifers (Kolotelo et al. 2001; Bonner 2008). In most cases, the seedlots from any one facility were collected within 1 year or within just a few years of each other, and likely stored using similar protocols, making the comparisons of seedlots from within each individual cooperating facility particularly strong. There might be slight differences in storage protocols (which may have been modified over time) among facilities, but these minor differences were not expected to impact the germination results to any major extent. One group of seedlots, the 20 whitebark pine seedlots from the USDA Forest Service's Institute of Forest Genetics (IFG, Pacific Southwest Research Station) had been stored in a walk-in cooler (1–3 °C) until 2009 (the year prior to initiation of this germination test), when they were moved to freezer storage. The 1996 United States Department of Interior Bureau of Land Management (USDI BLM) seedlot had been in and out of refrigerator and freezer storage, and a notation about the general lack of proper handling and storage was provided with the seed.

Approximately 100 seed (82–111) were used for each seedlot, except for two seedlots with only 50 seeds available and the BLM seedlot with 225 seed. Seed weight (g) was

Table 1 Number of seedlots, years of cone collections, and germination percentage by groups that provided whitebark pine seed

Source[a]	State/ province of origin[b]	# Individual parent lots	# Bulked seedlots	Collection year(s)	Mean germination percentage (min–max)
USFS IFG	CA	20	0	1985	63.6 (14.0–96.0)
USFS Region 1	ID, MT	5	0	1992–1997	41.0 (3.2–76.0)
USFS Region 5	CA, NV	10	1	1994	44.7 (4.0–83.2)
USFS Region 6 DGRC	OR, WA, WY	7	1[c]	1996–2003, 2009[d]	34.8 (6.5–51.5)[e]
USFS Region 6 JHSN	OR	0	1	1993	71.3
USFS Region 6 BSE	OR	0	2	1999	24.2 (0.0–48.5)
USFS RMRS	WY	0	1[c]	1996	75
USDI BLM	ID	0	1[e]	1996[f]	0
British Columbia	BC	1	2	1978, 1995	9.3 (3.2–20.6)
Alberta	AB	0	1	1986	0
Total		43	10	1978–2009[d]	47.7 (0.0–96.0)

[a] USDA Forest Service (USFS) groups included the Institute of Forest Genetics (IFG, Pacific Southwest Research Station), Northern Region (Region 1), Pacific Southwest Region (Region 5), Pacific Northwest Region (Region 6) John Herbert Stone Nursery (JHSN), Bend Seed Extractory (BSE), Dorena Genetic Resource Center (DGRC), and Rocky Mountain Research Station (RMRS). Also contributing seed were the USDI Bureau of Land Management (BLM), British Columbia (BC Ministry of Forests, Lands and Natural Resources Operations), and Alberta Tree Improvement and Seed Centre

[b] AB refers to Alberta, and BC refers to British Columbia in Canada. CA, ID, MT, NV, OR, WA, and WY refer to California, Idaho, Montana, Nevada, Oregon, Washington, and Wyoming, respectively, in the United States of America

[c] Same 1996 Shoshone National Forest collection, stored at two separate USFS facilities since 2001

[d] September 2009 collection used as control, put into stratification in October 2010, $\sim$1 year after collection (following a year of freezer storage at -16 °C), and excluded from calculation of statistics in the table

[e] Mean excludes the Shoshone National Forest collection. If it were included, the DGRC average would be 41.3%

[f] Seed sent in six separate bags representing seed from different cones, etc. (summarized together here)

taken for each sample prior to stratification (and is reported on a 100-seed basis), except for the seven Region 6 DGRC seedlots, which used the weight of a 100-seed sample measured in the years when the seed were originally extracted from the cones.

IFG seedlots

This trial included one unique group of whitebark pine seedlots (received from the USDA Forest Service's IFG) collected from Ball Mountain on the Klamath National Forest (Siskiyou County) in northern California. Cones were collected from 20 different parent trees in this population in September 1985. This collection provided an opportunity to examine within-population variation of seedlots collected in the same year from one location and stored under identical conditions. Germination data of these 20 lots were also available from 1985 when the freshly collected seed was sown for a study to look for major gene resistance to white pine blister rust. In that test, 28 seed per seedlot were sown at IFG,

and a short stratification period (<60 days) was used. Records noted germination as of 18 November 1985 (for seed received at IFG on 21 September).

X-rays of seed and prior germination data were not available for most seedlots. However, for the IFG seedlots, additional seed were later requested. Seed of 19 of the 20 seedlots from the 1985 Ball Mountain population collection were received and x-rayed in 2015. The x-rays provided baseline information on filled seed percentage to compare with the 2011 germination results. The x-rayed seed were assigned to one of five categories: (1) empty seed, (2) embryo filling <25% of seed corrosion cavity, (3) embryo filling 26–49% of cavity, (4) embryo filling 50–75% of cavity, and (5) embryo filling >75% of cavity. These represent a slight modification of the categories used in an earlier study of whitebark pine seed at DGRC (Berdeen et al. 2007).

Stratification and germination monitoring

Stratification and germination followed standard Dorena Genetic Resource Center protocols for whitebark pine (Riley et al. 2007). Stratification for whitebark pine seedlots began in mid-October 2010, except for two seedlots (one from Alberta, and the Shoshone National Forest bulklot from RMRS) that arrived later and were stratified beginning on 16 December using the same protocol. The whitebark pine seedlots were placed in individually labeled mesh bags, soaked for 24 h in 1% H_2O_2, rinsed, and then soaked for 24 h in water. The mesh bags were secured with binder clips and hung using the clips on dowels in covered plastic tubs. The tubs were placed in warm stratification at 10 °C for 30 days, then at 1–2 °C for 90 days. All seeds were rinsed once per week with water during stratification, and moldy seeds rinsed with 1% H_2O_2. A 90-day cold stratification (1–2 °C) period was used for foxtail pine, which began November 22 for three Region 5 seedlots, and November 29 for the seedlot from RMRS.

Following stratification, the whitebark pine seed were scarified on 17 February 2011 by abrading the seed coat with a sanding machine using 100-grit sandpaper at the radicle end (approximately 1 mm back from the tip). Whitebark pine seed that cracked their seed coats during stratification were not scarified. Under past germination protocols, scarification had been previously found to be useful for whitebark pine (Burr et al. 2001), but as with most other pine species, no scarification was recommended, and in this trial none was performed for foxtail pine. On 17 February, stratified seed of both species (except the two whitebark pine seedlots that were received late) were placed on moistened blotter paper in 10 cm × 10 cm clear plastic boxes in a germinator set to 16 °C night/18 °C day with a 12-h photoperiod. All seed for each seedlot were placed in a single box. The two late-arriving whitebark pine seedlots were placed in the germinator in late April following stratification, using the same protocol.

Seeds were monitored for germination two to three times a week for a period of four weeks; the count of germinated seed per seedlot was recorded February 20 through March 17, 2011. For the two late arriving whitebark pine lots, germination was monitored from April 25 through May 12. A seed was considered germinated once the radicle had emerged and was approximately 2 mm long. Germinated seed were discarded, and no growth or vigor assessments were done, except for three seedlots which were sown and included in a blister rust resistance trial. Following the monitoring period, up to 10 non-germinated seed per lot were cut and examined on 25 March (58 seed were cut for the BLM seedlot), and any late germinating seed were also recorded on this date. Cut seed were classified as viable or non-viable based on appearance. Seed which were classified as non-viable typically had no embryo, lacked endosperm, or exhibited degradation (such as soft or mushy

endosperm or embryo). Seeds that were considered viable had endosperm fully occupying the seed coat and embryos that appeared fully developed and occupying the cavity; there was no obvious macroscopic physical indication of why the seed did not germinate within the monitoring period. The dissection occurred following stratification and the germination period, and the seed were fully imbibed.

Germination percentage for each seedlot was calculated as [(number of seed germinating)/(number of seed in trial)] × 100. In addition to overall germination percentage, a graphical comparison was used, similar to that used by Berdeen et al. (2007), to examine the speed of germination of various ages of stored seed relative to seed stored for only a year (2009 seedlot).

Results

The seed of the three foxtail pine from the 1994 collections showed excellent germination, averaging 95.1%, with seedlots ranging from 90.2 to 99.0% germination. A majority of the seed in each of these seedlots germinated during the stratification period (61.2–68.7% germination). The seed from the 1997 collection, of unknown provenance and obtained in 2002 from a seed dealer, had no germinants, and during the seed dissection, the endosperm was noted as 'mushy' (not solid and firm).

In contrast to the foxtail pine, only 7 of the 53 whitebark pine seedlots had any seed germinate during stratification (only 1–3 seeds per seedlot). The 53 whitebark pine seedlots averaged 48.1% germination, with seedlots ranging from 0 to 96.0%. All but three of the 53 whitebark pine seedlots had at least one germinant (Fig. 1). The three seedlots (all bulk collections) that had no seed germinate were from different organizations and collection years, one a 1999 collection stored in Oregon (Bend Pine Seed Extractory), the second a 1986 seedlot that was from Alberta, and the third a 1996 BLM seedlot from Idaho. In contrast another 1999 bulk seedlot from the Bend Pine Seed Extractory had 48.5% germination.

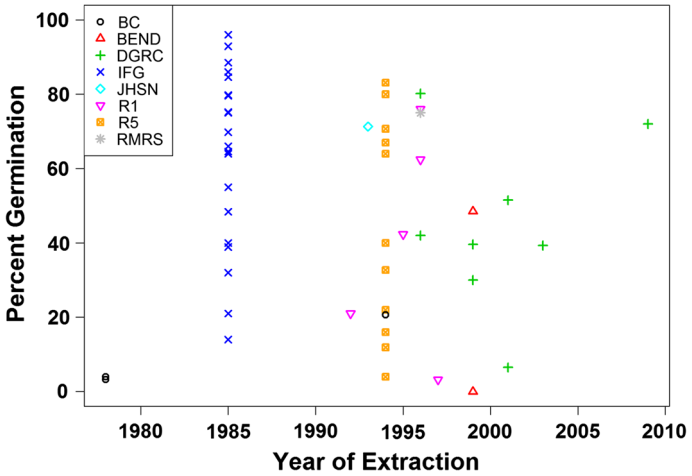

Fig. 1 Whitebark pine germination percentage in 2011 for each family or bulk lot and collection year combination. *Markers* with the *same color* or *shape* indicate the eight organizations and the seedlots they provided. Organizations with no germinants are excluded (Alberta and USDI BLM). (Color figure online)

The recent 2009 collection, a seedlot from a single parent tree from the Fremont-Winema National Forest in Oregon used as a control, had 72.0% germination. The 2009 control lot showed higher germination than the other six older seedlots also from Region 6 (6.5–51.5%) but had lower germination than 13 of the older seedlots from other areas, including 7 of the 20 seedlots from the 1985 IFG collection (Fig. 1). The speed of germination for the 2009 control seedlot was faster than that of the seedlot group means from any of the other 11 older collection years (Fig. 2a). However, on an individual seedlot basis, the speed of germination of several of the older seedlots, including some from the IFG 1985 collection, were comparable (or faster) to the 2009 seedlot (Fig. 2b).

There was high variability in germination percentage among seedlots stored at a common facility for each of the four USFS groups that contributed several seedlots. For example, the 11 Region 5 seedlots, collected in 1994 at various California locations, varied from 4.0 to 83.2% germination (Fig. 1; Table 1). Germination averaged 41.0, 44.7, 34.8 and 63.6% for Region 1, Region 5, Region 6 DGRC, and IFG, respectively (Table 1), and was very low for all three seedlots from British Columbia (Fig. 1; Table 1). The mean germination percentage of the seedlots from IFG was higher (by nearly 20%) than that of any group where more than one seedlot was available (Table 1). However, the bulk Shoshone NF seedlot stored separately at two facilities since 2001 had similar germination capacity in this 2011 test (80.2 and 75.0%).

IFG seedlots

One group of 20 whitebark pine seedlots, all collected from the same geographic area (Klamath National Forest, California) in 1985, averaged 63.6% germination in the 2011 test, with individual seedlots ranging from 14.0 to 96.0% germination (Fig. 1). The rate of germination of some of these older seedlots appeared comparable or even higher to the 'fresh' 2009 control seedlot (Fig. 2b). Two of the seedlots from this collection showed >90% germination even after 25 years in storage (Fig. 1). Seed from these seedlots had also been germinated for a 1985 blister rust resistance trial within months of seed collection. However, there was little relationship between the 1985 germination percentage and the 2011 germination percentage ($r = 0.028$, $p = 0.91$), and in most cases the germination in 2011 was much higher than in 1985 (Fig. 3). The mean germination percentage was 49.5% for 1985 versus 63.6% in the 2011 test. There was also no evidence that

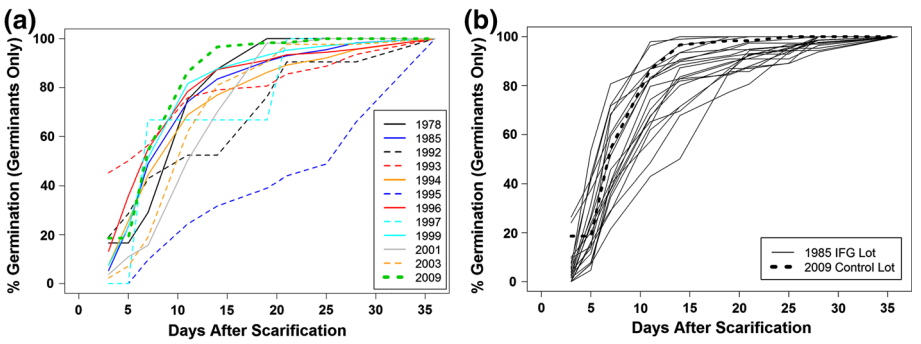

Fig. 2 Speed of germination: whitebark pine germinant germination percentage shown at different times post scarification for **a** all seedlots averaged within each of the 12 collection years (*left*) and **b** individual 1985 IFG seedlots and the 2009 control (*right*)

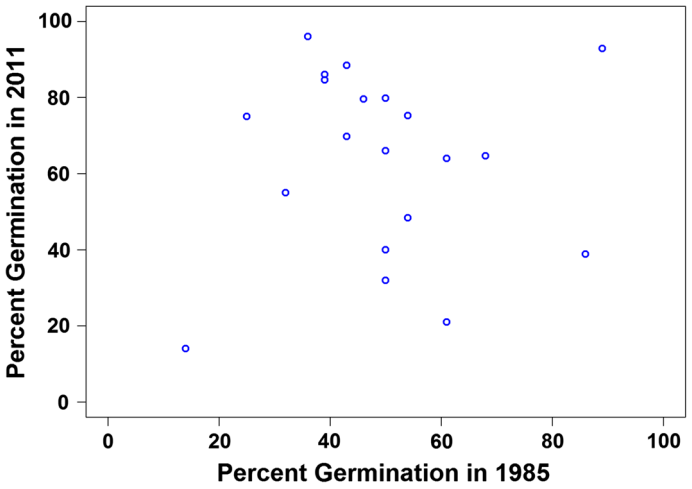

Fig. 3 Whitebark pine germination percentage shown for germination testing in 1985 and 2011 for 20 IFG lots collected in 1985 ($r = 0.028$, $p = 0.91$)

germination in 2011 was correlated with seed weight for this group (Fig. 4). There was suggestive but inconclusive evidence of a positive correlation between seed weight and germination in 1985 ($r = 0.40$, $p = 0.077$), but this relationship was much weaker with the heaviest seedlot excluded ($r = 0.21$, $p = 0.38$) (Fig. 4).

The x-rays of 19 of the 20 IFG lots indicated that the seedlots varied from 0 to 22 (mean = 6.2) in the percentage of empty seed. However, the length of the embryo can vary in filled seed, and only 7.9% of the seed had embryos filling >75% of the corrosion cavity (varied from 1 to 32% among the 19 lots). The percentage of seed with the embryo filling >50% of corrosion cavity averaged 57.9% for the 19 seedlots and varied from 26 to 93% among parent trees. The germination percentage of some of the IFG seedlots was greater than would be predicted based on the fraction of each seedlot that had the embryo filling

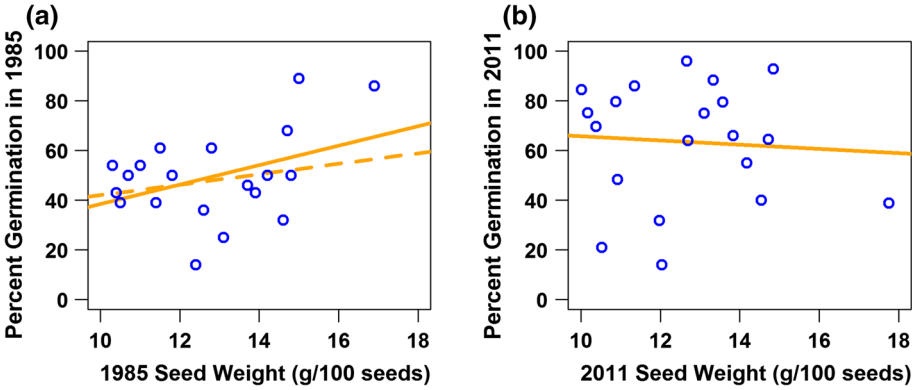

Fig. 4 Relationship between seed weight of 20 individual tree seedlots collected in 1985 from one population, and **a** 1985 germination percentage (fresh seed) ($r = 0.40$ and $r = 0.21$, solid line (all points) and dashed line (excluding highest seed weight), respectively), and **b** 2011 germination percentage ($r = 0.07$)

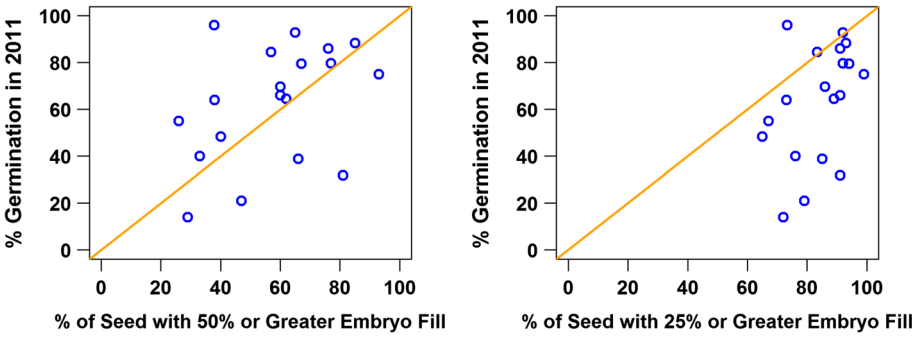

Fig. 5 Whitebark pine germination relative to the percentage of seed with the embryo filling at least 50% of the corrosion cavity (*left*) or at least 25% of the corrosion cavity (*right*)

>50% of the corrosion cavity, while the germination percentage was generally less than would be predicted if embryos filling greater than 25% of the corrosion cavity were the criteria for judging seed as viable (Fig. 5).

Dissection of samples of the non-germinated seed of both species showed several trends. In some cases, the seed was mushy and was totally non-viable; in others, an embryo was present and was sometimes green; in others no embryo was observed in the cavity. Thirty-nine of the 51 whitebark pine lots that had seed cut at the end of the germination period had 50% or more of the sampled seed rated as potentially viable, while ten seedlots had 20% or less seed rated as viable, including one lot with zero viable. The BLM lot had only 1 of 58 cut seed rated as viable.

Discussion

Adaptive genetic variation and gene conservation

Adaptive genetic variation in a species provides the basis for survival and evolution in the face of biotic and abiotic threats (Hamrick 2004; Schaberg et al. 2008). Non-native diseases and insects, as well as the changing climate, threaten many species in their native habitat. For example, although most whitebark pine and western white pine (*P. monticola* Dougl. ex D. Don) are very susceptible to white pine blister rust, there is genetic variation in the susceptibility to this non-native disease, and resistant parents are being identified. These resistant parents can be utilized as seed sources or for breeding and establishing seed orchards for reforestation and restoration (Sniezko et al. 2007, 2011a, 2014).

Climate change as well as non-native insects and diseases will continue to cause major disruptions to forest ecosystems, leading to potential functional or complete extinction of some tree species. Mitigation of the negative impacts of these factors through active management may be possible, in at least some cases, but it involves many considerations, and in all cases optimizing the potential success of restoration, or reforestation, of a species will require careful consideration of the current and future appropriate genetic seed sources to use (Dumroese et al. 2015; Jacobs et al. 2015; Stanturf 2015; Ledig and Kitzmiller 1992). Genetic strategies are needed to counter the threat posed by climate change and to maintain some forest species and ensure future healthy forest ecosystems (Ledig and Kitzmiller 1992; Dumroese et al. 2015; Jacobs et al. 2015; Stanturf 2015). For many tree

species, including those of high concern such as whitebark pine and foxtail pine, long-term storage of seed can provide a relatively inexpensive method of preserving their genetic diversity (Ledig 1988). It can provide an essential backup reservoir of the genetic variation of these species in case the in situ populations of these species continue to decline and as efforts to facilitate the species recovery at some level are contemplated (Dumroese et al. 2015; Ledig 1986). The availability of genetically diverse seed and research derived from its use will increase the likelihood of success of management activities (Dumroese et al. 2015; Ledig and Kitzmiller 1992). The more complete the level of genetic variation conserved in the ex situ seed banks collected while the species is relatively intact, the higher the likelihood of the long-term potential of re-establishing a species on at least some sites. Strategies to retain species on the landscape, such as assisted migration programs, will rely on research using ex situ seed collections from different parts of the species range (Jacobs et al. 2015).

Some, perhaps most, current environments for species such as whitebark pine and foxtail pine may be altered significantly by climate change. As climate change continues or new biotic or abiotic threats emerge for species such as whitebark pine or foxtail pine, the seed resource can be used for studies to ascertain whether or not genetic resistance or tolerance to these factors exists within the species, or to establish provenance trials or other field plantings to examine what seed sources and environments are compatible under the changing climate. Based on these studies, land managers can use the most appropriate genetic sources of seed available as a basis to start restoration of the species or to begin a breeding program or seed orchard (Ledig and Kitzmiller 1992).

The relatively modest amounts of seed currently in storage are insufficient for range-wide or landscape level restoration but could be used in the most conducive environments for the species to establish small stands of trees that would be the future parent trees for more extensive seed collections. Several such plantings for whitebark pine have recently begun in Oregon and Washington, and the family (seed tree) identities of each of the seedlings in these plantings have been documented (Sniezko unpublished). These field plantings established with diverse seed sources also become important sentinels to warn of changes in the environment and whether the genetic variation is sufficient to maintain the species.

Some groups are contemplating (or have started) seed orchards of whitebark pine, using grafts of parent trees, or rust-resistant progeny selections, and these will likely play a primary role in ex situ conservation and producing large quantities of seed for future restoration efforts. Such seed production areas or orchards will be needed if large quantities of genetically diverse seed are needed for restoration or if the viability of seed in storage diminishes over time. However, meaningful levels of seed production from these efforts are likely one or many decades away, and the seed in storage may be the main source of diverse germplasm to use for restoration efforts or research until then, especially for the populations that have undergone the most dramatic recent declines.

In contrast with whitebark pine, at this point in time, seed storage is the primary genetic conservation effort underway for foxtail pine; few or no in situ or ex situ plantings have been undertaken to date. For this reason, knowledge of the viability of foxtail pine seed in long-term storage is paramount to be able to utilize it for any future restoration efforts. If the need arises, the seed from storage could be used to prototype small seed orchards to produce genetically diverse seed for future use, or for research to more fully understand the adaptive capacity of this species. Only a relatively few seedlots of foxtail pine have been examined for resistance to white pine blister rust, and the future availability of viable seed

would also help facilitate further work to develop seed sources with genetic resistance to white pine blister rust.

Germination of stored seed

Seed storage is a widely used, long-standing method of ex situ genetic conservation for many plant species. Although it has been documented that seed of some other conifers has retained viability for up to 60 years (see summary in Kolotelo et al. 2001; Krugman and Jenkinson 2008), to evaluate the utility of seed storage as a genetic conservation strategy more data are needed on how long seeds of each species can be stored and retain viability, including any variation that exists in storage potential among seed collected from different parent trees or different geographic areas within the range of the species. A recent large, concerted effort has gone into seed collection of whitebark pine and to a lesser extent foxtail pine for ex situ genetic conservation (Sniezko et al. 2011b) but without updated guidelines on how long seed of these species can be stored. This trial was undertaken to confirm whether at least some seed remained viable over one or more decades of storage, and it included the oldest known available seedlots of two North American high elevation white pine species, whitebark pine and foxtail pine. In this trial, nearly all of the seedlots had some germination, and several of the seedlots had very high percent germination (>90%).

The three foxtail pine seedlots collected in 1994 showed excellent germination in this trial. In a subsequent 2014 sowing of foxtail pine for a white pine blister rust resistance screening trial, seed of these three lots germinated well again, and the seedling vigor (as indicated by height and survival) of the 2-year old seedlings from these three seedlots was comparable to the other foxtail pine from more recent collection years (Sniezko, unpublished data). The fourth foxtail pine seedlot used in the 2011 study, a 1997 collection from RMRS storage, had no germination, and an examination of the cut seed indicated that they were not viable (the seed had embryos, but the tissue was very soft). Little is known about the history of this 1997 seedlot and whether the seed was originally of poor quality, handled poorly or deteriorated while in storage. This small sample indicates that at least some properly collected and stored foxtail pine seed can retain excellent viability for at least 20 years. The high percentage of seed that germinated during the 90-day stratification period suggests that future investigations are needed to determine if a shortening of the recommended stratification period for foxtail pine (Krugman and Jenkinson 1974) should be considered. Because seed storage is the current primary method of ex situ genetic conservation for foxtail pine, it will be important to continue to evaluate the viability of seed over time for this species.

There was generally a very wide variation in germination percentage among whitebark pine seedlots (Figs. 1, 4) that, unless otherwise noted, were stored under very similar or identical protocols. This wide range of variation in germination of whitebark pine seed (Fig. 1; Table 1) is higher relative to other conifer species collected and stored under similar protocols (Kolotelo et al. 2001). Only one of the tested seedlots had seed stored in two different facilities [a bulked seedlot collected from the Shoshone National Forest (WY) in 1996], and it had similar levels of germination between facilities in the DGRC test. This suggests that differences in initial seed quality or genetic variation in this seedlot affected the observed germination, and such factors are also likely the source of wide variation in germination among the other seedlots.

Three of the 53 whitebark pine seedlots had no germination. Documentation sent with these seedlots regarding previous testing (Bend Seed Extractory and Alberta) or seed

handling (BLM) suggested that the seed might not germinate well. The seed cut test performed on two of these seedlots at the end of the trial confirmed the poor condition of these seedlots relative to the more viable-looking seed remaining for many of the other seedlots. No observations were made on the non-germinating seed of the late sown Alberta seedlot. The low germination percentage for the three British Columbia seedlots (3, 4 and 20.6%) was not unexpected; a previous assessment of their low viability (3–32% germination) was noted in correspondence (Dave Kolotelo, personal communication).

The germination of the other 50 whitebark pine seedlots is encouraging and provides strong evidence that many seedlots of this species can retain viability in storage for at least 10–25 years. In addition, from the dissected samples of non-germinated seed at the end of the study, 39 of these seedlots appeared to have at least 50% viable-looking seed, suggesting that perhaps refinements in stratification and germination protocols might further improve the germination capacity. However, since initial germination capacity was not known for many of these seedlots, further studies will be needed to determine if any notable decline in germination capacity or seedling vigor occurs over time and how much it varies by parent tree.

The youngest whitebark pine seedlot (selected to use as a control), collected in 2009, had 72% germination, which was generally higher relative to older seed, but 14 of the older whitebark pine lots had greater germination than this 1-year-old lot. The germination percentage of this 2009 seedlot was good, but not as high as had been previously observed in at least some other fresh seed, including several of the IFG seedlots germinated in 1985 (Fig. 3). Seed of some conifer species store relatively well for at least several decades; however, there are differences among species and even seedlots within species, and further studies are needed to understand the deterioration that occurs for some species or seedlots (Kolotelo et al. 2001; Bonner 2008). Age of seed is only one factor that influences germination capacity; it can also be influenced by factors such as seed maturity, seed handling, and the stratification and germination protocols (Burr et al. 2001; Kolotelo et al. 2001; Bonner and Karrfalt 2008). There has also been concern that scarification of the whitebark pine seed may damage some seed, and its effectiveness may vary by the individual doing the scarification (Charlie Cartwright, personal communication).

Interestingly, some of the oldest available whitebark pine seed, the 20 seedlots from IFG collected in 1985, were stored for 24 years under slightly warmer temperatures (than freezer storage that is the more frequently used current protocol) and yet had the highest germination (63.6%) of any group of seedlots. These 20 seedlots from IFG were collected from one population in 1985 and thus provided an opportunity to examine the inherent variation in germination for seedlots collected from different parent trees in the same general location at the same time and handled the same way in storage. These seedlots varied widely in germination percentage in both 1985 and 2011 (Figs. 1, 3). A comparison of their germination in the year collected (1985) with that in 2011 (Fig. 3) showed little relationship between the germination of fresh seed and stored seed from those same seedlots tested 25 years later. This lack of relationship between the two trials might be partially explained by the much shorter stratification period used in 1985. Research since that time has indicated that a longer stratification period is needed to maximize germination (Burr et al. 2001), and DGRC used a 120-day stratification period for this trial. However, two seedlots from this group of 20 had much better germination in 1985 than in 2011 (Fig. 3); this suggests that the germination capacity of these two seedlots may have declined in storage, that a different stratification protocol might be needed for some seedlots, or that some seedlots may have had more damage from scarification than others. The rate of germination in 2011 also varied among these 20 seedlots, and some of the IFG

seedlots germinated as rapidly as the 1-year-old control (Fig. 2). Wide variation also existed in germination percentage between seedlots collected in each of the other geographic areas and stored at other facilities (Fig. 1), suggesting further study of the seed biology of whitebark pine and/or refinements to the stratification and germination testing protocols are needed.

For the 20 IFG seedlots, there also was no relationship between seed weight and percent germination in 2011. Previous work has examined similar relationships, and an effect of seed weight on germination capacity was found for short stratification periods when germination was relatively poor (Bower et al. 2011). However, for non-germinating seeds undergoing an additional, longer stratification, this relationship disappeared (Bower et al. 2011).

An examination of the x-rays of 19 of the IFG whitebark pine seedlots showed that they varied widely in percentage of seed in the four embryo classes and in percentage of empty seed. However, the corresponding germination data indicated that some seedlots with a higher percentage of seed in the smallest embryo class germinated well, so even some whitebark pine seed with very small embryos must have germinated. Some evidence of this was seen in a prior study (Berdeen et al. 2007). A future scheduled germination trial of these 19 seedlots using a new protocol (see discussion below) should provide additional information relating to the germination capacity of 31-year old whitebark pine from different parent trees and with different levels of embryo maturity. This new sowing will also allow us to examine seedling vigor of these whitebark pine stored for a prolonged period of time, something that was beyond the scope of the study we report in this paper. For seed storage to be an effective ex situ genetic conservation tool, seed must produce vigorous seedlings as well as retain good germination capacity.

One factor that may influence long-term whitebark pine seed viability is the widely different levels of embryo maturity that can be present in this species (Burr et al. 2001). The degree of embryo immaturity in whitebark pine can vary dramatically by year and by geographic area (Sniezko, unpublished) as well by parent tree within a population. This was noted not only in the IFG group of seedlots studied here but also in previous work at DGRC (Berdeen et al. 2007). For example, most of the whitebark pine seedlots collected in Oregon and Washington in 2011 for ex situ genetic conservation and blister rust resistance testing had relatively small embryos and showed relatively poor germination in initial sowing using the same stratification and germination protocols of this study (Sniezko unpublished). In the trial reported here, 29 of the whitebark pine seedlots had some late germinants (1–14 per seedlot, noted at time of seed dissection), along with a relatively large percentage of non-germinated seed rated as viable in the seed dissection. Thus, for some species, and notably for whitebark pine, it is important to note the condition of the seed at time of collection, and that stratification and germination protocols used may influence the results of the seed viability testing. Although protocols for many conifers are relatively well established, some species such as whitebark pine appear to require further development of stratification and germination protocols to optimize germination. It is also possible that differential protocols may be needed based on geographic origin of seed or its age.

The five whitebark pine seedlots with the lowest germination were from collections that had been noted as having low or suspect quality by their suppliers, emphasizing that care has to be taken at all stages from timing of cone collection to seed extraction to adequate protocols for storing seed. For properly collected and stored seed, the recent refinements and the newly adopted whitebark pine stratification and germination protocols adapted from work in Alberta (Lindsay Robb, personal communication), including a longer

stratification period, stratification in sand, and no scarification, are expected to increase germination levels (Riley et al. 2016; Haley Smith and Richard Sniezko, unpublished; Charlie Cartwright, personal communication).

For many of these seedlots, prior germination data or x-rays were not available or different germination protocols may have been utilized, so no assessment on whether there has been a decline in germination is presented here. However, the high germination of the foxtail pine and the high germination of at least some of the whitebark pine seedlots is encouraging. Some seed of most whitebark pine and foxtail pine seedlots retained viability for at least two decades in storage, extending the recommendations on storage time that were provided in the earlier guidelines (Krugman and Jenkinson 2008). Further trials and monitoring will be needed to examine whether the seeds of these species retain viability for the even more extended storage times that might be needed, or whether new seed collections (if available) will be needed or alternatively if planting of seed currently in storage will be required to establish parents of future seed crops (before the seed loses viability).

The cause of the wide range in the germination percentage among the whitebark pine seedlots cannot be ascertained from this trial. It may have been due to factors such as inadequate collection protocols, seed immaturity when harvested, improper handling or storage, the stratification protocol used in 2010, seed scarification damage, as well as possible inherent within-species genetic differences that would influence the longevity in freezer storage. The wide range in germination observed among whitebark pine seedlots collected from the same area has important management implications. As an example, a bulked seedlot of whitebark pine to be used for research or restoration that is expected to represent wide genetic variation for the species may effectively be much less genetically diverse due to highly differing germination rates from the seed from different parent trees. Knowledge of the germination level variation between the seedlots used in the bulked seedlot can be used to alter the composition of any bulked lot that is a composite of seed stored separately, allowing scientists or land managers to achieve the desired level of genetic diversity for their goals. Burr et al. (2001) also cautioned that for whitebark pine the genetic contribution from within a single seedlot might be influenced by the speed of germination of that seedlot and that there could be resulting ecological implications; thus including the early as well as later germinating seed may be warranted. Further investigation into how genetic variation from seed from each parent tree included in the bulked lot is affected by the seedlot's germination (percentage and speed) is warranted. Protocols to maximize germination, including uniformity (speed), would help to allay these concerns.

Due to the limited number of seedlots from each geographic source and other factors, we cannot not provide much evidence for geographic trends in germination of whitebark pine. However, in a more recent sowing of a provenance trial of 257 seedlots in British Columbia (BC), including 156 from BC and 99 from the U.S., the mean germination for the BC seedlots was 35 versus 78% for the U.S. seedlots (Charlie Cartwright, personal communication), suggesting there could be a geographic trend in germination and that seedlots from the far northern part of the species range may have less viability. The 78% germination for the 99 seedlots from the U.S. is impressive. A comparison of germination of the 81 seedlots in the BC provenance trial that were also previously sown at DGRC revealed a considerable differential in germination success between the two facilities, with the germination in BC being higher (Charlie Cartwright, personal communication). The stratification protocol used in Canada was slightly different than the one used in this study and included extra aeration in seed soaking and use of sand versus a naked stratification (Charlie Cartwright, personal communication), which is more similar to the protocol now adopted at DGRC, several years after the completion of the study being reported here

(Riley et al. 2016). Although the new stratification and germination protocols should improve the already positive scenario for storage of whitebark pine seed, it is still unknown at what point beyond perhaps two or three decades of storage that a serious decline in viability or seedling vigor may take place. Continued testing of seedlots at periodic intervals will be needed to ensure that germination and seedling vigor remain at a level sufficient for the goals of the gene conservation program.

Long-term seed storage as a method of ex situ genetic conservation is an important tool in tree species conservation. It complements other components of ex situ and in situ conservation. Due to costs and other factors, it may be the primary means of conserving the genetic variation of some species. However, an understanding for each species of the expected viability under different time periods of seed storage is essential. The refinements in seed storage, stratification and germination protocols being developed for a species such as whitebark pine provide the opportunity for more controlled analyses in the future, including a comparison of germination rates of fresh seed with that of seed stored for various time periods and will help us understand any differences in retention of seed viability while in freezer storage amongst different geographic sources or individual parent trees. These future investigations will help us understand how well we can generalize the results both within and across geographic areas and address management concerns such as retaining genetic diversity across the landscape for any future restoration efforts. Similar data will be needed for any species for which it has not been adequately accumulated.

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