Figure 1. Following the growing season, seedlings are moved into cold storage where they are labeled and organized. Come spring, the seedlings are removed and staged in a wax-lined cardboard box for shipping to customers. Photo by Kennedy Pendell, 2023.

Evaluating Cold Storage Effects on Big Sagebrush (*Artemisia tridentata*) Seedlings Through Seedling Quality Tests

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Abstract

The accumulation of chilling hours prior to cold storage and outplanting is essential for seedling cold hardiness, which allows seedlings to withstand cold temperatures. This project analyzed the effects of cold storage on outplanted big sagebrush (*Artemisia tridentata*) seedlings and performed electrolyte leakage and root growth potential tests. Big sagebrush seedlings with the highest survival had 500 chilling hours prior to cold storage, and electrolyte leakage tests showed this was a sufficient number of chilling hours for this species to become cold hardy prior to storage. Additionally, the fewer hours that big sagebrush seedlings spent in cold storage, the more successfully the seedlings grew roots in the root growth chamber. Determining the sufficient number of chilling hours that big sagebrush requires can aid nurseries in managing the storage of sagebrush, while understanding the relationship between cold storage and root growth potential can aid restoration professionals in estimating how well seedlings will grow when outplanted at restoration sites.

Introduction

Prior to European-American settlement, the big sagebrush ecosystem encompassed 156 million acres (63 million ha) throughout western North America (Boyle and Reeder 2005). This ecosystem supports 735 species (Remington et al. 2021) that are adapted to environmental conditions that include arid conditions during the summer and freezing temperatures during the winter. Currently, it is estimated only 50 percent of the big sagebrush species remain (Adler et al. 2018, Boyle and Reeder 2005). The loss of big sagebrush (*Artemisia tridentata*), an arid/semi-arid shrub, is of particular concern to range ecologists (Innes 2019).

Land use changes are the primary driver of the loss of big sagebrush ecosystems. These changes are a direct result of human activities and development and their interactions with other complex factors, such as altered fire regimes, invasive species, conifer encroachment, drought stress, and livestock grazing. Through these changes, competitive species, such as cheatgrass (*Bromus tectorum*), can become the dominant plant in this ecosystem. Given the fire-prone nature of the ecosystem, an increase in the fire frequency may limit the reestablishment of big sagebrush (Baker 2006).

Restoring big sagebrush ecosystems is a priority for organizations that include the U.S. Department of the Interior's Bureau of Land Management, the U.S. Department of Agriculture's Forest Service, the Idaho Fish and Game, and the World Center for Birds of Prey in Idaho. A primary goal of this restoration is increasing the distribution of big sagebrush across the landscape, which is accomplished through outplanting to supplement natural regeneration. Nurseries will play a crucial role in growing these seedlings, and one such nursery is the Forest Service's Lucky Peak Nursery in Boise, ID. This nursery currently produces about 2.5 million seedlings annually, which are a mix of conifer and shrub species grown as bareroot or container stock.

Lucky Peak Nursery follows the standard practice of placing seedlings into cold storage, which allows for flexibility in nursery management and plays an important role in the quality of seedlings produced (figure 1). Seedlings kept overwinter are stored in a freezer at 28 °F (-2 °C) and remain in a dormant state until conditions are favorable for outplanting (Overton et al. 2013).

However, cold storage conditions are unlike the environmental conditions that seedlings are exposed to in the field or in the greenhouse. Coolers are cold and dark, temperatures are low and constant, and there is high humidity (Ritchie 1987). When in cold storage, seedlings lose the ability to produce carbohydrate reserves through photosynthesis and instead consume carbohydrates through respiration to survive. The low temperature of cold storage reduces the rate of respiration, thereby prolonging the carbohydrate reserves and allowing the seedlings to survive longer (Ritchie 1987).

Cold hardiness is the capacity of plant tissue to withstand exposure to freezing temperatures (Herriman et al. 2012) or the minimum temperature at which a certain percentage of a seedling population will survive or withstand a given level of damage (Haase 2011). It is an essential physiological state that seedlings require to survive winter. Seedlings accumulate hardiness as photoperiod shortens, soil moisture decreases, and temperatures drop during the fall (Herriman et al. 2012). Cold hardiness can be measured by tracking photoperiod, accumulated chilling hours, or a combination of the two; chilling hours refer to the duration of time at which a seedling has been exposed to temperatures at or below 42 °F (5.5 °C) (Ritchie 2004). Federal nurseries use cold hardiness as an indicator of stress tolerance for seedlings.

Cold hardiness is critical for successful outplanting because it has been linked to higher survival and growth in the field (Haase et al. 2016). While long-term cold storage effects on seedlings have been studied for many conifer species, primarily Douglas-fir (*Pseudotsuga menziesii*) (Ritchie 1987, Simpson 1990), limited research has been conducted on the viability of big sagebrush seedlings in relation to time spent in cold storage. Measuring accumulation of chilling hours prior to placing seedlings in cold storage would give Federal nurseries a parameter to determine if seedlings are dormant and have enough carbohydrate reserves to survive in cold storage.

The chilling hour requirement for all seedlings at the Lucky Peak Nursery is 350 hours prior to cold storage (Nelson 2022, Ritchie 2004). Currently it is assumed that this requirement is the same for shrub species and other conifer species. Developing recommendations for how nurseries can prepare seedlings for cold storage may increase survival of outplanted big sagebrush and the success of restoration projects.

Methods

For this project, seedlings were sourced from the Lucky Peak Nursery. At Lucky Peak Nursery, seedlings are placed in either refrigerated storage between 33 and 35 °F (1 and 2 °C) or freezer storage at 29 °F (-2 °C). The Lucky Peak Nursery tracks seedling cold hardiness by calculating chilling hours and aims for seedlings to have a minimum of 350 chilling hours prior to cold storage to reach optimal cold hardiness (Ritchie 2004).

Plant Materials

Lucky Peak Nursery provided the seeds used for this experiment, which were collected at a site near the nursery in 2018. Seeds were sown into six Beaver Plastics Styroblocks (112 series) in March 2021. The Styroblocks were placed with another big sagebrush seedling lot for the growing season, known as a crop (figure 2). An overhead irrigation boom regularly misted the crop until germination occurred. Irrigation occurred at 70 percent of field capacity based on block weight (Dumroese et al. 2015). The crop was fertilized every three irrigation cycles with Peters Professional Conifer Finisher 4-25-35 with magnesium and YaraLiva CALCINIT 15.5-0-0 at 50 ppm.

Seedlings remained in Styroblocks until December 2021. At time of extraction, seedlings received exactly 506.4 chilling hours, exceeding the standard operational target at Lucky Peak of 350 chilling hours. Seedlings were extracted from Styroblocks and placed randomly into seedling bags in bundles of 10. Seedling bags were then placed horizontally into a lightly waxed box with a plastic box liner (figure 3). A total of 448 seedlings were extracted and placed into a freezer at 29 °F (-2 °C).

Figure 3. Once seedlings are extracted from the blocks, they are placed into thin plastic bags and packed into wax-lined boxes for cold storage. Photo by Kennedy Pendell, 2023.

Planting Site

The planting site was located at the Lucky Peak Nursery, where a small, 17-acre (6.9-ha) fire occurred in September 2020 and cleared most of the old endemic shrubs and native perennial forbs and grasses. One year after the fire, rush skeletonweed (*Chondrilla juncea*), bulbous bluegrass (*Poa bulbosa*), cheatgrass, medusa head (*Taeniatherum caput-medusae*), common storks-bill (*Erodium cicutarium*), and other invasives dominated the area. The site harbors wintering mule deer and offers habitat for ground-dwelling birds, small mammals, and predatory species such as coyotes and bobcats. These site characteristics made this an ideal location for a big sagebrush seedling survival study due to its similarities to big sagebrush restoration sites.

Figure 2. Big sagebrush crop used for this project in the greenhouse at Lucky Peak Nursery. Photo by Kennedy Pendell, 2022.

Planting

Beginning on March 21, 2022, 20 seedlings were randomly selected and pulled out of the freezer every 2 weeks and planted (table 1). To avoid root damage that could occur when the seedlings were planted, 2 days prior to planting they were placed in a walk-in cooler set to 33–35 °F (1–2 °C). In the planting site, seedlings were planted 3 feet apart in rows in a south-to-north orientation. Each outplanting had a designated row: outplanting (OP) 1 to OP17, and seedlings in each lot were numbered 1 to 20 in a south-to-north orientation. A drill with a 2-inch auger bit was used to drill the holes. Seedlings were placed in the holes with no portion of the plug still visible above the soil profile

Table 1. Dates of 17 outplantings of sagebrush seedlings, 2022

Outplanting	Date	Outplanting	Date
OP1	Mar. 21	OP10	July 25
OP2	Apr. 4	OP11	Aug. 8
OP3	Apr. 18	OP12	Aug. 22
OP4	May 3	OP13	Sept. 4
OP5	May 16	OP14	Sept. 18
OP6	May 31	OP15	Oct. 3
OP7	June 14	OP16	Oct. 16
OP8	June 27	OP17	Oct. 29
OP9	July 9		

Each planting included 20 sagebrush seedlings. The 8 months of plantings spanned both the ideal outplanting windows in the spring and fall, and the less-than-ideal time during the summer.

and soil compacted around the soil plugs to avoid air pockets and frost heaving near the roots (figure 4) (Shaw et al. 2015). The planted site encompassed 0.07 ac (0.2 ha) and included 17 rows of planted seedlings following 17 outplantings that spanned from March to October 2022.

Data Collection

Root Growth Potential

Every 2 weeks, 20 seedlings were randomly pulled from the freezer for outplanting. Two seedlings from each batch were randomly selected for testing root growth potential (RGP) and used as a control for each outplanting (figures 5 and 6). RGP is a useful indicator for seedling vigor and quality, and Lucky Peak Nursery uses this method to analyze a seedling's capability to produce new roots under ideal growing conditions.

Figure 4. Seedling from outplanting 3 (OP3) a few months after planting. Photo by Kennedy Pendell, 2022.

The two seedlings thawed in a walk-in cooler set between 33 and 35 °F (1 and 2 °C) for 2 days before being placed into the RGP chamber. Seedlings were monitored over 14-day time periods. The RGP chamber stayed in a temperature-controlled building at roughly 59 °F (15 °C) for the entirety of this study. Supplemental lighting provided 10 hours of light a day to promote growth. At the end of each 2-week period, new roots greater than 0.2 in (5 mm) in length were recorded. The seedlings' RGP was rated on a scale of 0–4 using the Lucky Peak Nursery's protocol (table 2).

LT_{50} Testing and Frost Tolerance

Cold hardiness, also known as frost tolerance, is the ability of a plant to withstand freezing temperatures that can damage the plant cell tissues (Atucha Zamkova et al. 2021). The frost tolerance of seedlings can be tested using electrolyte leakage assessments. Of the available methods to conduct electrolyte leakage testing, the freeze-induced electrolyte leakage (FIEL) test was used.

FIEL testing uses seedling samples that are frozen to various decreasing freezing temperatures (Nelson 2022). At each benchmark temperature, the electrolyte concentration in the water is measured to determine the percentage of electrolyte leakage from the plant tissue. This value is used as a metric of tissue damage caused by freezing temperatures. Based on these measurements, the

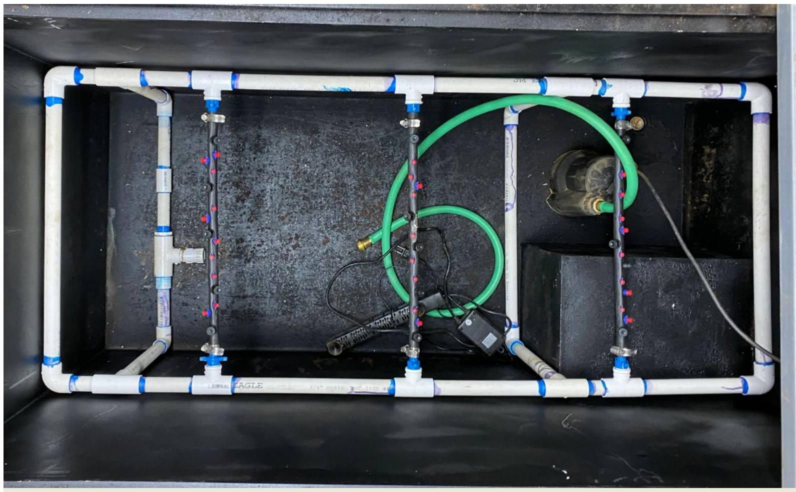

Figure 5. Root growth potential chamber used at Lucky Peak Nursery. Photo by Kennedy Pendell, 2023.

Figure 6. Seedling from OP17 in the root growth potential chamber. Photo by Kennedy Pendell, 2022.

lethal temperature for 50 percent (LT_{50}) of the population can be determined.

Samples consisted of nine replicates of randomly selected seedlings or clippings. These samples were randomly selected from when seedlings had no time in cold storage, at the time of the first outplanting, and from two outplantings in November 2022. Samples were placed in seedling bags and placed in a Styrofoam cooler with a freezer pack to maintain seedling vigor during the shipping process. Samples were overnighted to the University of Idaho's Pitkin Forest Nursery, which performed the FIEL tests immediately after arrival.

Leaves from each seedling sample were randomly selected, however, leaves with visible damage were avoided. Leaves were cut into 0.4-in (1-cm) segments and put into 20-mL vials with 10 mL of deionized water. The vials were then capped and placed into a programmable freezer set to decreasing temperatures of 19, 7, -6, -18, -31, and -40 °F (-7, -14, -21, -28, -35, and -40 °C). One sample set was removed at each temperature benchmark and set into a refrigerator to thaw at 36 °F (2 °C). Once samples were thawed, electrolyte concentration was measured with a Mettler Toledo SevenEasy conductivity meter. Next, all samples were completely killed in an autoclave followed by a secondary conductivity measurement. These two measurements were used to calculate the relative electrolyte leakage for each temperature (Nelson 2022).

Table 2. The Lucky Peak Nursery rating system for root growth potential

Index	Rating	Observation
0	Dead	No new root initiation
1	Poor	1–5 new roots at least 0.2 in (5 mm) in length
2	Fair	6–10 new roots
3	Good	10–20 new roots
4	Excellent	Greater than 20 new roots

Data derived from these tests determined the LT_{50} through plotting the index of injury against temperature and assuming a linear relationship between the two values. A lower LT_{50} value (more negative) indicates that a seedlot has higher cold hardiness. A higher LT_{50} value (more positive) indicates a seedlot has lower cold hardiness (Haase 2011). An upper threshold cold hardiness value for most conifer species is -4 °F (-20 °C), which can be assumed for shrub species like sagebrush (Nelson 2022, Simpson 1990).

To represent results from the LT_{50} tests, graphs were fitted with a sigmoidal (Gompertz) curve using SigmaPlot 14.5. The index of injury was created using direction from Flint et al. (1967) and data received from the LT_{50} tests conducted at the University of Idaho's Pitkin Forest Nursery.

$$\text{Index of injury } (T) = 100 \text{ x } (RT - R0) / (1 - R0)$$
$$RT = L1T / L2T$$
$$R0 = L1C / L2C$$

where

T is temperature

$L1T$ and $L2T$ are the initial and final leakage values for a sample exposed to temperature (T)

$L1C$ and $L2C$ are the corresponding values measured from respective control samples

Electrolyte leakage data starts at 0 percent and values are spread between 0 and 100 percent by adjusting the leakage values from totally injured samples as suggested by Lim et al. (1998):

$$\text{Percentage-adjusted injury } (T) =$$
$$(\text{index of injury } [T]/\text{index of injury } [T\ lowest]) \text{ x } 100$$

where

index of injury (T) is the value obtained at respective freeze-treatment temperature

index of injury (T *lowest*) is that obtained at the lowest test temperature (-31/-49 °F (-35/-45 °C)) (Nelson 2022)

Results

The root growth observed in the RGP chamber was mixed (figure 7). For OP1, whose seedlings spent the least amount of time in cold storage, the RGP index rating was 0.5. This low RGP could be due to poor seedling health of the random samples, a malfunction of the root growth chamber water system, or even the environmental controls of the building the chamber was in. The same assumptions could be made for OP3 and OP7.

The RGP results show successful root production (aside from OP1, OP3, and OP7) until OP15. Root production of these seedlings show that when these seedlings are planted in ideal growing conditions, the seedlings should be successful in the field.

For seedlings stored for 10 to 11 months (OP15 through OP17), there was a significant reduction of root growth with half of the population not accumulating roots at all. The mortality of seedlings reached 50 percent beginning with OP15. These results suggest that seedlings can spend about 9 months in cold storage before 50 percent mortality is reached.

Staff conducted four LT$_{50}$ tests over the course of the experiment (table 3). The first LT$_{50}$ test was conducted on a random sample of nine seedlings from the entire seedling population on the date of extraction, and these seedlings had 506.4 chilling hours. This test resulted in the lowest LT$_{50}$ value at -19.59 °F (-28.66 °C), indicating the seedlings had sufficient cold hardiness prior to cold storage. This value is a 2.55 °F (-16.36 °C) difference from the highest LT$_{50}$ value, which was found for OP1 with the second test. The second LT$_{50}$ test was conducted on a random sample from the entire seedling population at the time of OP1. This test resulted in an LT$_{50}$ value at 9.86 °F (-12.3 °C), which indicated the seedlings did not exceed the upper threshold cold hardiness value at the time of outplanting.

On November 30, 2022, the third and fourth LT50 tests were conducted on clippings taken from OP1 and OP5. OP1 had an LT$_{50}$ value of -9.4 °F (-23.0 °C) and OP5 had -11.99 °F (-24.44 °C). Based on these values, OP1 had less cold hardiness at the time of outplanting on March 30, 2022, than OP5 that was outplanted on November 30, 2022. OP5 had more cold hardiness than OP1 both before and after planting. However, each of these outplantings did reach the upper threshold of the cold hardiness value of -4 °F (-20 °C) (figure 8).

Table 3. The lethal temperature where 50 percent of the sampled seedlings died (LT$_{50}$)

Test date	Outplanting (OP)	LT$_{50}$ °C	LT$_{50}$ °F
Dec. 8, 2021	Initial pack, no storage	-28.66	-19.59
Mar. 30, 2022	OP1 (before planting)	-12.3	9.86
Nov. 30, 2022	OP1 (after planting)	-23.0	-9.4
Nov. 30, 2022	OP5 (after planting)	-24.44	-11.99

The LT$_{50}$ value is used to determine how cold hardy a seedling population is at the time of testing.

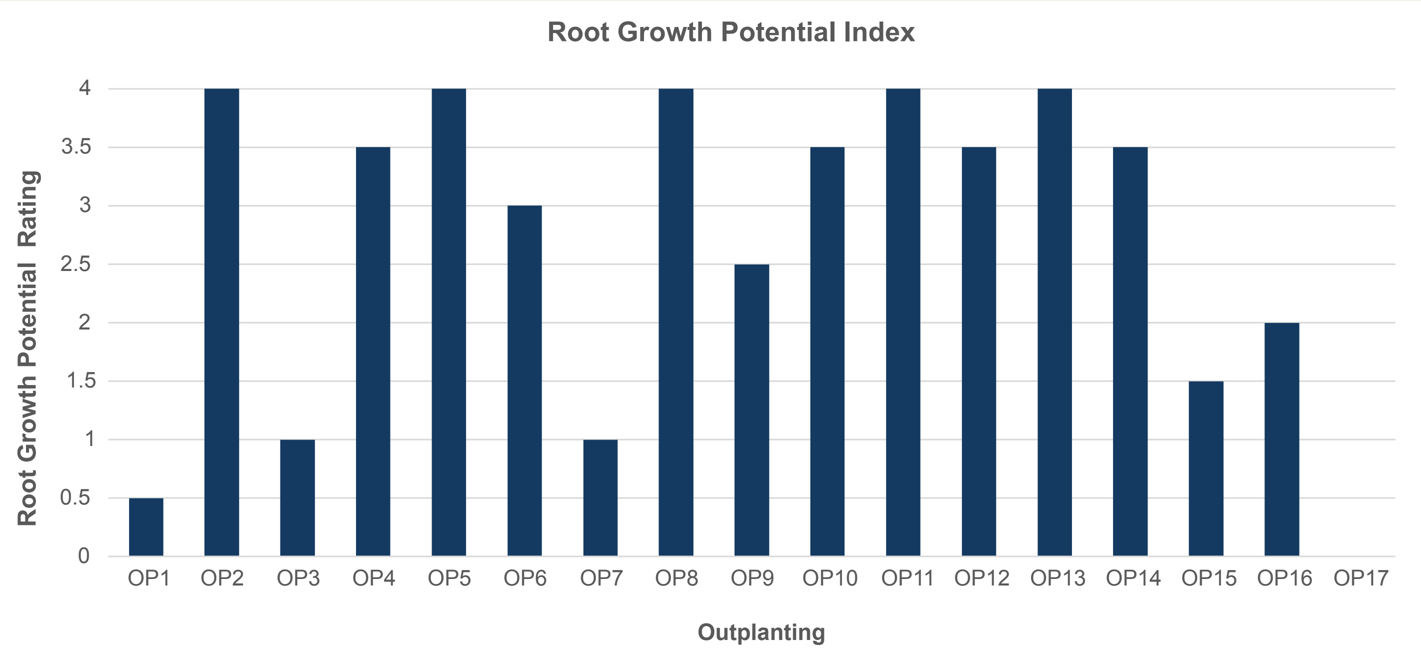

Figure 7. The root growth potential index rating for each of the outplanted seedlings. See table 2 for further details.

Discussion

This study analyzed each of the steps taken by Lucky Peak Nursery to grow big sagebrush seedlings for restoration projects and paired it with a cold hardiness experiment. Based upon the results, it is possible to develop recommendations for nursery production and outplanting.

The viability of seedlings through OP14, which occurred on September 18, shows that sagebrush seedlings can be kept in cold storage for up to 9 months and successfully survive outplanting. Apart from OP1, OP3, and OP7, 100 percent of seedlings for each outplanting produced new roots until OP15. Thereafter (OP15 to OP17), only 50 percent of seedlings produced new roots. It can be speculated from these results that the less time seedlings spend in cold storage, the higher survival rates outplantings will have if planted during ideal growing conditions. While seedlings remain viable with 9 months in cold storage, it is advised that seasonality of selected planting sites is taken into consideration, because temperature and soil moisture are still driving factors of seedling success.

Freezer conditions throughout the study did not change for these seedlings. They were kept at a constant 28 °F (-2 °C). There is always a concern for seedlings to mold when placed into overwinter storage; seedlings may also begin to grow in storage. Neither of these occurred during this study. Loss of leaf color on the seedlings did occur the longer seedlings spent in storage.

Cold hardiness testing of the big sagebrush seedlings confirmed that the seedlings were sufficiently cold hardy after 506.4 chilling hours. At the time of storage, the LT$_{50}$ value for a given sample of the entire population was -19.59 °F (-28.66 °C). At the time of OP1, the LT$_{50}$ value for a given sample of the entire population was 9.86 °F (-12.3 °C). This confirms that FIEL test measurements are characteristic of low LT$_{50}$ values during a growth phase, and high when seedlings are dormant (Nelson 2022). In this study, seedlings were not placed into cold storage directly when seedlings reached 350 chilling hours. It is possible that the seedlings were sufficiently cold hardy at 350 hours, but the seedlings in this study surpassed this chilling requirement. However, this study shows that 500 chilling hours is sufficient for sagebrush seedling cold hardiness prior to cold storage.

Traditionally, LT$_{50}$ plots should show an inflection point in the data where temperature maximum (T max) is found. The T max was not found for the first, third, and fourth LT$_{50}$ tests. This could be due to the programmable freezer at the University of Idaho having a minimum temperature of -40 °F (-40 °C), where most reach -58 °F (-50 °C). It could also be due to the methods used to find LT$_{50}$, assuming that 100 percent mortality of seedlings is reached at the maximum temperature of the programmable freezer.

Sagebrush seedling cold hardiness, which was determined by FIEL testing and finding the LT$_{50}$, confirmed that the seedlings were sufficiently cold hardy at the time of extraction prior to cold storage. Future studies could attempt to perform an electrolyte leakage test closer to 350 hours to further confirm that is enough chilling hours for not only conifers, but sagebrush seedlings as well.

While the LT$_{50}$ data from this study confirm that the minimum chilling requirement Lucky Peak Nursery uses for its seedlings is sufficient, the study did not investigate how long-term cold storage affected all outplantings using this method. Further research could perform electrolyte leakage tests after storage for all outplantings. The data derived from this research

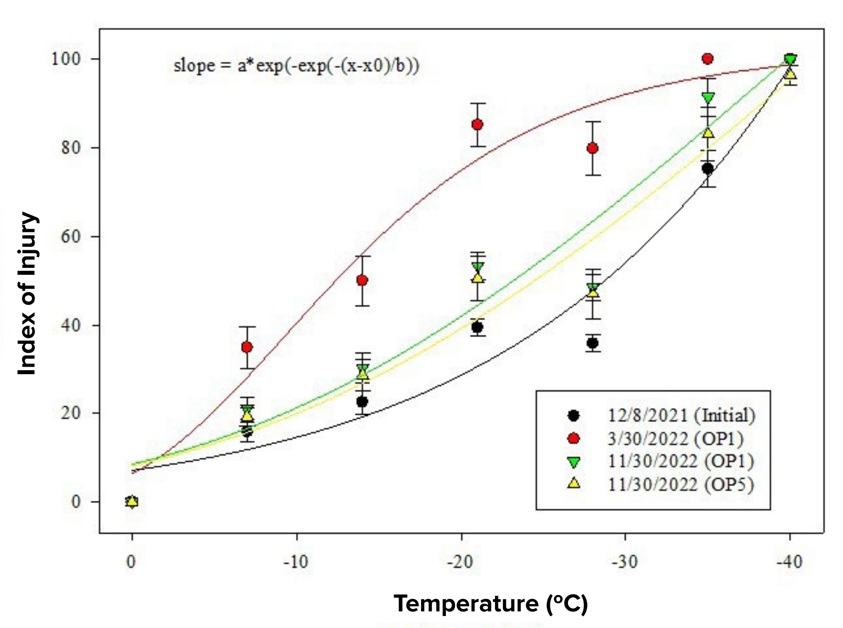

Figure 8. The relationships between temperature and index of injury (determined with electrolyte leakage) of big sagebrush seedlings at different storage dates (outplanting dates). Sigmoidal model fit is included.

could be used to analyze how cold storage influences cold hardiness of seedlings over a prolonged period. This would provide nurseries and their clients with insights on survival of sagebrush seedlings in relation to cold hardiness after cold storage.

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