IN VITRO REGENERATION, ROOTING, AND CLONING OF *ARTEMISIA TRIDENTATA*

by

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The following individuals read and discussed the thesis submitted by Rachael Barron, and they evaluated their presentation and response to questions during the final oral examination. They found that the student passed the final oral examination.

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DEDICATION

I would like to dedicate this work to my son, Logan Page. I have been in school, in some capacity, almost his entire life. He has been so supportive and patient through it all.

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ABSTRACT

Artemisia tridentata (big sagebrush) is an ecologically important shrub found in western North America. *In vitro* techniques can be applied to big sagebrush for the purpose of studying gene function, genotypic and phenotypic plasticity studies, cloning, genotypic preservation, and restoration. I performed experiments to develop an indirect organogenesis protocol to regenerate whole Wyoming big sagebrush plants from leaf explants. Callus formation frequency was 88% (±4.0%) in leaf explants cultured on medium containing 0.5 mg/l BAP and 1.0 mg/l NAA. Shoot formation frequency was variable between replicates and was the highest when callus tissue was cultured on medium containing 1.5 mg/l BAP and 0.1 mg/l NAA, 37% to 80%. I tested several auxin treatments to induce root formation and concluded the best to be 0.5mg/l IBA, which yielded 42% to 60% rooting. Taking into account all these variables, I estimate the total regeneration efficiency to range between 14% to 43% on this set of treatments. This protocol was also applied to basin big sagebrush. Callus formation was 100% in leaf explants. Shoot formation was 34% (±14.6%), but shoots exhibited a hyperhydric phenotype and were not transferred to root induction medium. The *in vitro* regeneration protocol developed is a crucial element that would be required to transform big sagebrush using molecular approaches. Experiments were also conducted to determine the feasibility of shoot tip and nodal cuttings to develop adventitious roots *in vitro*. This method can provide genetically identical material much faster than *in vitro* regeneration. Adventitious root formation in Wyoming big sagebrush cuttings cultured on two media

types was inconsistent, ranging from 10% in some experiments to 80% in others. Limited success was achieved in nodal cuttings cultured on modified MS medium containing auxin and cytokinin 12.5% (±5.6%). No root formation was achieved in mature plant tissue collected in the field. Results indicated that genotypic influences were likely more responsible for variations in rooting than the medium or vessel conditions tested. Cloning experiments in basin big sagebrush further supported this notion. All material for these experiments came from half-sibling individuals that was maintained separately throughout the course of the experiments. Some half-siblings formed no adventitious roots on any treatments tested whereas others had high rates of formation on all treatments. Further studies, utilizing exogenous PGRs, such as auxins, may provide more successful adventitious root formation in shoot tips from both big sagebrush subspecies.

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LIST OF ABBREVIATIONS

BAP	6-benzylaminopurine
CIM	Callus Induction Medium
CO_2	Carbon dioxide
IBA	Indole-3-butyric acid
IAA	Indole-3-acetic acid
NAA	Naphthalene acetic acid
O_2	Oxygen
PGR	Plant Growth Regulator
SIM	Shoot Induction Medium
RIM	Root Induction Medium
TDZ	Thidiazuron

CHAPTER ONE: *IN VITRO* REGENERATION OF *ARTEMISIA TRIDENTATA*

Introduction

Artemisia tridentata (big sagebrush) is a common shrub in western North America that contributes to the development of a heterogeneous landscape and provides habitat and forage for local animals (Davies et al., 2007; Aldridge & Boyce, 2007). Due to the critical roles of *A. tridentata* in sagebrush habitats, there is considerable interest in re-establishing this shrub following fires. In particular, one subspecies is of much interest, *A. tridentata* ssp. *wyomingensis* (Wyoming big sagebrush). This subspecies usually occupies xeric locations, which makes re-establishment particularly difficult (Boyd & Obradovich, 2014). Development of new experimental tools to study Wyoming big sagebrush may ultimately provide insights to improve the re-establishment of this valuable shrub.

The subspecies *Artemisia tridentata* ssp. tridentata (basin big sagebrush) is also ecologically important. Basin big sagebrush is often found in well-drained fertile soils. In contrast, Wyoming big sagebrush tend to be present in shallow, dry and rocky soils (McArthur & Plummer, 1978). While genomic data is limited, it is known that Wyoming big sagebrush is exclusively a tetraploid subspecies. Basin big sagebrush is primarily a diploid however, tetraploid individuals can be found (McArthur & Sanderson, 1999). Genetic variation resulting from both intra and interspecific hybridization is also observed in subspecies of big sagebrush (Bajgain et al., 2011).

A vegetative propagation protocol for big sagebrush would be valuable. This protocol could be used to obtain clones from individual plants with desirable traits and contribute to the understanding of phenotypic and genotypic plasticity. *In vitro* plant regeneration can be used to obtain clones. Moreover, *in vitro* regeneration would be an important step towards transforming sagebrush using molecular techniques, which would facilitate the analysis of gene function in this species. More understanding of the genes responsible for successful environmental adaptations may allow for the selection of plants better suited for certain environmental conditions.

Plant *in vitro* culture has a wide range of applications. Micropropagation has been utilized to produce large amounts of desired clonal lines for agricultural crops (Habtamu, 2016), ornamentals (Preil, 2003), genotype preservation (Tavazza et al., 2015), and reforestation (Barberini et al., 2015). Meristem culture is also an effective method to obtain pathogen-free plants (Bhatia, 2015). Medicinally important secondary metabolites have been isolated from plants grown in tissue or liquid cell culture. Plants bioengineered and grown *in vitro* have also been used to produce pharmaceutical compounds (Espinoza-Leal et al., 2018). *In vitro* tissue propagation and preservation has been used for endangered plant species that produce few seeds or seeds with deep dormancy (Pence, 2011). The plants produced using these methods can be reintroduced into areas where they have become extinct or to increase wild populations (Chandrika & Ravishankar, 2009).

I*n vitro* regeneration begins with an explant, a small portion of plant tissue. Explants can be derived from a variety of plant tissues, including stem, leaf, cotyledon, seed, and root. Explant tissue type is one of the many variables that can affect the

regeneration potential within a species (Yildiz, 2012). Explants are often cut on the ends to create a wound. This wound, with the addition of auxin and cytokinin, promotes the formation of a callus (Ikeuchi et al., 2019). This mass of undifferentiated cells can then be transferred to medium containing different concentrations and combinations of plant growth regulators (PGRs) such as cytokinin, auxin, abscisic acid, and gibberellin to induce the formation of plant organs. The process of regeneration through a callus intermediate is known as indirect organogenesis. Some studies have found they can skip this callus phase and go directly from wounded explant tissue to shoot formation, which is known as direct organogenesis (Hussain et al., 2012).

Some plants, such as many cultivars of lettuce (Armas et al., 2017) and coniferous species (Bonga & Klimaszewska, 2010), are not responsive to *in vitro* regeneration. A species is deemed recalcitrant after large multifactorial studies are unsuccessful or yield a low organogenesis success rate. The cause of recalcitrance is poorly understood. New methods to quantify the endogenous PGRs in plant species may be a valuable tool in designing protocols for *in vitro* regeneration (Erland et al., 2017). Observation-based modeling combined with genetic algorithms is also becoming a tool utilized in the optimization of *in vitro* regeneration protocols (Mridula et al., 2018).

Designing *in vitro* regeneration methods in a novel plant species can be an extremely laborious and time-consuming process. There are several studies of *in vitro* regeneration in other plants in the *Artemisia* genus such as *A. pallens* (Alok et al., 2016), *A. annua* (Dangash et al., 2015) and *A. vulgaris* (Sujatha & Kumari, 2007). Many of the media components used in this study were selected from the most successful media components found in these other species of *Artemisia*. Turi et al, (2014) demonstrated

that big sagebrush germplasm could be maintained in tissue culture. While the successful formation of adventitious roots was observed in shoot clumps excised from seedlings, individual shoot tips did not form adventitious roots even with the addition of PGRs. To date, and to the best of my knowledge, there are no publications on the *in vitro* regeneration through organogenesis of Wyoming big and basin big sagebrush. The overall objective of this study was to develop a protocol to regenerate big sagebrush explants *in vitro*.

Materials and Methods

Seed Sterilization

The Wyoming big sagebrush seeds used in this study were a generous gift from Dr. Joseph Sirotnak (Bureau of Land Management), who collected them from populations of Wyoming big sagebrush within the Morley Nelson Snake River Birds of Prey National Conservation Area in southwestern Idaho.

Damaged seeds and other plant materials were separated from intact seeds under a dissecting microscope and intact seeds were rinsed under running water for approximately two hours. I then surface sterilized the seeds in a 0.5% (v/v) sodium hypochlorite solution containing a surfactant (0.1% TritonX-100) for 10 minutes. Seeds were then rinsed in sterile water for 20 minutes. Fresh sterile water was replaced every five minutes for a total of four washes.

Growth Medium and Conditions

I placed the sterilized seeds into magenta GA-7 vessels containing a sterile growth medium. This growth medium was prepared with ½ strength Murashige & Skoog (MS) macro and micronutrients (Murashige & Skoog, 1962) containing ½ strength

modified Gamborg (B5) vitamins (Gamborg, 1966), 1% (w/v) sucrose, and 3 g/l phytagel. One ml/l PPM (Plant Preservative Mixture, Plant Cell Technology) was added to the medium to prevent potential contamination. Twenty seeds were placed per Magenta GA-7 vessel which were then placed under fluorescent lights. Seedlings were grown at room temperature (~23°C) with a 16 h photoperiod.

Callus Induction

Material for callus induction was taken from 60-day old seedlings. I tested leaf, cotyledon, and root tissue for its ability to form callus *in vitro*. The PGR formulation to induce callus was adapted from an *in vitro* regeneration publication of *A. annua* (Dangash et al., 2015). Callus induction medium (CIM) was prepared with ½ strength MS macro and micronutrients (Murashige & Skoog, 1962) containing ½ strength modified B5 vitamins (Gamborg, 1966), 1% (w/v) sucrose, 6 g/l agar, 0.5 mg/l 6-benzylaminopurine (BAP), and 1.0 mg/l naphthalene acetic acid (NAA). The same medium formulation without the addition of BAP and NAA was used as a control treatment.

Explants were taken from several seedlings and randomized prior to being placed on CIM. Root tissue explants were approximately 1-1.5 cm long. Explants from leaf and cotyledon tissue were approximately 1-1.5 centimeters long and 2-5 millimeters wide (**Figure 1.1 A**). The explant material was cut on both ends to facilitate callus formation. Leaf and cotyledon explants were placed on media abaxial side down.

Five replicates for each explant source were placed on CIM and control medium. Ten root explants were cut for each replicate. Five leaf and five cotyledon explants were cut for each replicate. Treatments were placed in a dark incubation chamber at 23°C.

Callus formation was recorded after four weeks. Explants that formed callus were then transferred to different media to induce shoot formation.

Shoot Induction

After callus formation, I transferred the callus to shoot induction medium (SIM). The PGR combinations tested were based on media formulations that have been successful for shoot formation in *A. annua* and *A. vulgaris* (Dangash et al., 2015; Sujatha & Kumari, 2007). Four media treatments were tested. A control, that had the same composition as the CIM (modified MS medium with 0.5 mg/l BAP, and 1.0 mg/l NAA), and three media that have the same base composition of CIM with different concentrations of BAP, NAA, and gibberellic acid (GA_3) (**Table 1.1**). On each of the four media, I placed four explants with callus derived from cotyledons, five explants with callus derived from leaves, and ten explants with callus derived from roots. Plates were incubated at ~23°C with a 16 h photoperiod. The number of calli with shoot formation was recorded after 4 weeks.

Further characterization of the variability within treatment two (**Table 1.1**) was conducted in two subsequent experiments using only leaf explants. For each experiment, I cut 250 leaf explants, which were approximately 1 cm long and 2-5 mm wide. Twenty-five explants were placed abaxial side down on the same CIM medium that was used in experiment one. Plates were then incubated in the dark at 23°C for 4 weeks.

Leaf explants that formed callus were then transferred to SIM containing 1.5 mg/l BAP and 0.1 mg/l NAA. Plates were stored at ~23°C with a 16 h photoperiod. The number of calli with shoots was counted and recorded four and six weeks after placement on SIM for the first experiment and four and eight weeks for the second experiment. Calli

were transferred to SIM every four weeks. Shoots that formed were subsequently transferred to root induction medium (RIM).

Table 1.1 Shoot Induction Media Treatments

Treatment	BAP (mg/l)	NAA (mg/l)	GA_3 (mg/l)
1	1.5	0.05	0.0
2	1.5	0.1	0.0
3	1.0	0.0	3.0
4 (control)	0.5	1.0	0.0

Root Induction from Leaf Explants

To induce the formation of adventitious roots on the regenerated shoots, I tested three media formulations. All three media consisted of the same modified MS medium used previously but differed in the PGRs added (**Table 1.2**). The same medium without auxin was used as a control, while the other two treatments contained the auxin NAA or indole-3-butyric acid (IBA) at 0.5 mg/l. Two replicates of 25 shoots were used per treatment. Before transferring the shoots, I trimmed off callus tissue around the base from where the shoot was forming, but without removing all the callus tissue as this would have been detrimental to the morphology of the regenerated shoots.

Table 1.2 Root Induction Media Treatments: Leaf Explants Experiment 1

Treatment	NAA (mg/l)	IBA (mg/l)
1	0.5	0.0
2	0.0	0.5
3 (control)	0.0	0.0

Four and eight weeks after transferring the shoots to the three media treatments, the number of shoots with roots was counted. Shoots without root formation were transferred to fresh medium after four weeks. Shoots with root formation were transferred from the treatment plates into Magenta GA-7 vessels containing the same growth medium that was used for germinating and growing seedlings.

Based on the results of the first root induction experiment, I conducted an additional experiment to test other PGR treatments. For this experiment, the four treatments tested were all composed of ½ strength MS macro and micronutrients (Murashige & Skoog, 1962), ½ strength modified B5 vitamins (Gamborg, 1966), 1% (w/v) sucrose, and 3 g/l phytagel, but differed in the concentration or type of auxin added as listed in **Table 1.3**. Indole-3-acetic acid (IAA) was added as a treatment for this experiment. The gelling agent was changed in this experiment due to observation that it was difficult to remove rooted shoots from agar without damaging the roots. As in the previous experiment, I transferred regenerated shoots with a small amount of callus tissue into the RIM treatments. Four replicates of six shoots were included per treatment. Shoots with root formation were transferred from the treatment plates into magenta GA-7 vessels containing the modified MS growth medium.

Table 1.3 Root Induction Media Treatments: Leaf Explants Experiment 2

Treatment	NAA (mg/l)	IBA (mg/l)	IAA (mg/l)
1	0.5	0.0	0.0
2	0.25	0.0	0.0
3	0.0	0.5	0.0
4	0.0	0.0	0.5

Root Induction from Root Explants

One hundred root explants from 60-day-old seedlings grown *in vitro* were cut and placed on the same CIM used in previous experiments. Explants were approximately 1.5 cm long. Plates were incubated in the dark at ~23°C for four weeks.

Explants that formed callus were transferred directly to a medium to try to induce root formation. For this experiment, both ½ strength MS macro and micronutrient + ½ strength modified B5 vitamins and full-strength MS macro and micronutrient + full strength modified B5 vitamins were tested. Phytagel was tested in addition to agar. The addition of the auxins NAA and IBA was also tested (**Table 1.4**).

Table 1.4 Root Induction Media Treatments: Root Explants

Treatment	MS strength	Gelling agent	NAA (mg/l)	IBA (mg/l)
1	Half	Agar (6 g/l)	0.0	0.5
2	Half	Agar (6 g/l)	0.5	0.0
3	Full	Phytagel (3 g/l)	0.5	0.0
4	Full	Agar (6 g/l)	0.5	0.0

Shoot Regeneration from Leaf Explants of Regenerated Plants

An experiment was conducted to observe if there were higher shoot formation rates in explants that came from plant material that had already been regenerated. In all previous experiments, explants were derived from seedlings that were germinated i*n vitro*. For this experiment, regenerated shoots that had rooted were removed from the media containing PGRs and grown in Magenta GA-7 vessels containing growth medium for approximately eight weeks. I cut a total of 150 leaf explants and distributed them equally in 10 plates

containing the same CIM that was used in all previous experiments. Plates were stored in the dark at ~23°C for four weeks.

After callus formation, explants were transferred to SIM containing 1.5 mg/l BAP and 0.1 mg/l NAA (Treatment 2). The time on the different media and the growing conditions were the same as in previous experiments. The number of calli with shoot formation was recorded after four weeks on SIM. This four-week data was compared to the shoot formation frequency observed in leaf explants derived from 60-day-old seedlings germinated *in vitro*.

Basin Big Sagebrush Regeneration

The most successful regeneration protocol observed in Wyoming big sagebrush was applied to basin big sagebrush. I surface-sterilized the seeds following the same protocol and germinated them in Magenta GA-7 vessels with growth medium. Leaf explants were taken from six-week-old seedlings and cut in the same manner as Wyoming big sagebrush explants. Twenty leaf explants were sampled from five half-sibling individuals. Unlike the previous *in vitro* regeneration experiments, explants were not randomized prior to being placed on media treatments. Ten leaf explants per plate were placed abaxial side down on the CIM that contained 0.5 mg/l BAP and 1.0 mg/l NAA. Plates were stored in the dark at ~23°C for four weeks.

After four weeks, the callus tissue was transferred to two different SIM. One medium consisted of ½ strength MS macro and micronutrients containing ½ strength modified B5 vitamins, 1% (w/v) sucrose, 6 g/l agar and 1 ml/l PPM 1.5 mg/l BAP and 0.1 mg/l NAA, while the other had the same components except for the MS salts and Gamborg vitamins, which were present at full strength. Ten explants from each half-sibling were

placed on each treatment. Plates were stored at ~23°C with a 16 h photoperiod. The number of calli with shoot formation was recorded after four weeks.

Data Analysis

Wyoming big sagebrush explants from several seedlings were randomized prior to being placed on treatments. Explants from Basin big sagebrush were separated per half-sibling individual. Callus formation in the different explant types was analyzed by an ANOVA. Callus formation in leaves over three experiments and shoot formation in different explant types were analyzed using a Kruskal-Wallis analysis of variance with a Dunn post hoc analysis. An ANOVA was performed on root formation frequency data with a Tukey-HSD post hoc analysis. Basin big sagebrush regeneration data were analyzed using a two-way ANOVA. All data were analyzed in R.

Results

Callus Induction

There was no callus formation on the control treatment that lacked PGRs. In contrast, callus formation occurred in the three explant types tested on the PGR treatment (**Figure 1.2**). Differences between explants were not significant ($p = 0.28$) and the average frequency of callus formation was 76% (± 8.8%) for cotyledons, 88% (± 7.2%) for leaves (**Figure 1.1 B**), and 94 % (± 3.6%) for roots.

The rate of callus formation in leaves was significantly different over three experiments (Kruskal, $p = 0.036$) (**Figure 1.3**). The average rate of callus formation in leaf explants in the first experiment was 88% (±7.2%). In the second and third experiment, the average rate of callus formation in leaf explants was 88% (±7.7%) and 100%, respectively. A Dunn post hoc analysis showed that there was a significant

difference between the first and third experiments (p = 0.03672) and the second and third experiments (p = 0.0400).

Shoot Induction

The frequency of shoot formation among all treatments was compared by the type of explant **(Figure 1.4)**. Root explants did not form shoots on any treatment. A Kruskal-Wallis analysis of variance did not result in a significant difference between explant type among all shoot induction treatments (p = 0.23). Despite the lack of significance, leaf explants had an average shoot formation frequency on all treatments that was numerically higher than cotyledon explants. More importantly, leaves were identified as the most efficient explant type due to the greater amount of tissue availability in starting material as compared to cotyledons.

Callus tissue derived from leaf and cotyledon explants successfully formed shoots when exposed to treatment 2 and treatment 3 (**Table 1.1**). Treatment 2 resulted in the highest frequency of shoot formation from leaf and cotyledon calli at 80% and 50%, respectively. (**Figure 1.5**) (**Figure 1.1 C**). Callus tissue derived from roots did not form shoots on any treatment.

Treatment 2 was selected as the SIM to move forward with for the *in vitro* regeneration protocol. Due to the limited amount of leaf explant material available for testing in this first experiment, further characterization of the variability within the treatment was explored. The average rate of shoot formation from leaf calli in this subsequent experiment was 23.3% (± 6.3%) at four weeks and 38.1% (± 10.6%) at six weeks. A high rate of variability was observed between replicates (**Figure 1.6**).

In the third experiment, variability in shoot formation was observed at four and eight weeks after being placed on the SIM. At four weeks the average shoot formation frequency was 31.1% (± 4.6%) and at eight weeks the average rate of shoot formation was 37% (± 4.4%) (**Figure 1.7)**.

Root Induction

The first experiment was designed to test the auxins NAA and IBA (0.5 mg/l) for their ability to initiate root formation. The same medium without the addition of auxin was used as a control treatment (**Figure 1.8**). Shoots on the IBA treatment had an average root formation frequency of 42% (± 8.5%). Shoots on the NAA treatments had an average root formation frequency of 78% (± 2.8%). No roots formed on the control treatment. A statistically significant difference between treatments was observed (ANOVA, $p = 0.0015$). A Tukey-HSD post-hoc analysis revealed a significant difference between all three treatments (IBA: Control, $p = 0.0013$, NAA: Control, $p = 0.0080$, NAA: IBA, $p = 0.012$).

No morphological differences were observed in the shoots on the RIM treatments. Shoots that formed roots were removed from the treatments and placed into Magenta GA-7 vessels with the same medium that was used to grow seedlings. During this transfer, I observed that roots frequently broke off from the shoots when trying to remove them from the plates. To reduce this damage in the subsequent root formation experiments, I used phytagel (3 g/l) as the gelling agent instead of agar for the second RIM experiment.

In the second root formation experiment, I tested the auxins NAA, IBA and IAA. After six weeks in culture the average frequency of root formation for the NAA treatment

at 0.25 and 0.5 mg/l was 18% (± 9.2%) and 49% (±12.2%), respectively (**Figure 1.9**). The average frequency of root formation for the 0.5 mg/l IBA treatment was 62% (± 10.4%) and 46% (± 9.1%) for the 0.5 mg/l IAA treatment. The differences observed between these four treatments were not significant (ANOVA, $p = 0.12$).

At the time of the transfer to RIM, all the regenerated shoots had a normal phenotype. However, after four weeks in the rooting medium, I noticed clear differences in shoot phenotype between the different media (**Figure 1.10**). In the IBA medium, shoots maintained a normal phenotype. However, in the other media the shoots were highly compacted, difficult to separate and appeared hyperhydric. Thus, even though the different media yielded similar frequencies of root formation, the IBA medium was superior to the other media tested based on the phenotype of the regenerated plants.

Regeneration from Root Explants

Root explants formed callus on the modified MS medium with 1.5 mg/L BAP and 0.5 mg/L NAA at a high frequency (>90%) in all experiments. This explant source failed to form shoots or roots on any of the four treatments tested.

Shoot Regeneration from Leaf Explants of Regenerated Plants

Leaf explants derived from plant material that was already regenerated had an average frequency of shoot formation of 62% (± 5.1%) after four weeks on SIM. This shoot formation frequency was significantly higher than the shoot formation frequency of leaf explants derived from randomized seedlings ($p = 0.00019$) (**Figure 1.11**).

Basin Big Sagebrush Regeneration

The morphology of Basin big sagebrush seedlings germinated and grown *in vitro* was like that of Wyoming big sagebrush seedlings grown under the same conditions. Also,

like for Wyoming big sagebrush, the formation of callus from leaf explant was high, reaching 100% callus formation frequency in all five of the half-siblings tested.

From leaf calli, shoot formation occurred on the two SIM treatments at similar frequencies, 34% (±14.6%) and 26% (±12.5%) for the half and full-strength modified MS media, respectively (p = 0.46). In contrast, I observed a significant difference in the frequency of shoot formation between leaf explants derived from each half-sibling individual (p = 0.034); these frequencies ranged from 0 to 90% (**Figure 1.12**). Although shoot formation was observed in four out of the five half-siblings, the shoots exhibited hyperhydricity. This phenotype made it difficult to collect shoots from callus and the former were not tested for their ability to form roots.

Discussion

Through the experiments conducted in this study, I developed a protocol to regenerate Wyoming big sagebrush from leaf explants *in vitro*. The regeneration protocol involved three main steps: formation of callus from leaf explants, differentiation of shoots from leaf calli, and rooting of these shoots. Callus formation was consistently higher than ~90% in a culture medium with 0.5 mg/l BAP and 1 mg/l NAA. Regeneration of shoots from callus was more variable, and in the medium selected, which contained 1.5 mg/l BAP and 0.1 mg/l NAA, ranged from 36.9% to 80%. Albeit to a lesser extent, I also observed variability during root induction. As proven by the rates of rooting and the overall phenotype of the seedlings, the best rooting medium had 0.5 mg/l IBA. In this medium, the frequency of root formation varied between experiments from 42% to 60%. By multiplying the rates of callus or organ formation at each step in the regeneration protocol, the estimated overall rate of regeneration ranged to 14% to 43%.

The media and concentration of PGRs tested in my study were primarily selected based on *in vitro* regeneration and micropropagation results from other *Artemisia* species. The PGR type and concentration used for the CIM was used by Dangash et al. (2015) for the *in vitro* regeneration of leaves in *A. annua*. The authors reported a 100% callus formation frequency on this treatment. I achieved a 100% callus formation frequency in leaf explants in the third experiment conducted. The callus formation frequency in my first and second experiment was 88% (±7.2%) and 88% (±7.7%) respectively. The callus formation may have been lower in initial experiments due to the lack of optimization of the technique.

The PGR type and concentration used in shoot induction Treatments 1 and 2 (**Table 1.1**) were also successful in the Dangash et al. study in *A. annua* (2015). The authors reported an average shoot formation frequency in Treatment 1 and 2 of 83.6% and 26% respectively. The authors did not report the variability observed between replicates. I observed no shoot formation in treatment 1 and an 80% frequency of shoot formation in treatment 2. Further experiments to characterize the variability within this treatment, resulted in final shoot formation frequencies of 38.1% (±10.6%) and 36.9% (±4.4%). Treatment 3, which contained the PGRs BAP and GA_3 was adapted from *in vitro* regeneration in *A. pallens* and *A. vulgaris* (Alok et al., 2016; Sujatha & Kumari, 2007). Gibberellic acid was used in *A. pallens* to promote shoot elongation and in *A. vulgaris* to promote multiplication. I observed the frequency of shoot formation in this treatment to be 40%. Shoots appeared similar in size and morphology to the Treatment 2.

The RIM treatments I tested were also successful in both the study in *A. annua* and *A. pallens* (Dangash et al., 2015; Alok et al., 2016). Dangash et al. reported an

average frequency of root formation of 85.8% in regenerated shoots on 0.5 mg/l NAA. When I tested this treatment, I found the average frequency of root formation of 78% in my first experiment and 49% in my second experiment. The treatment I decided to move forward with, 0.5 mg/l IBA, had an average root formation frequency of 59.6% in the *A. annua* study and 67.6% in *A. pallens.* I found an average frequency of root formation of 62% in this treatment.

The Wyoming sagebrush seedlings in my study showed marked differences in regeneration capacity between leaf and root explants. Although root explants formed callus, this callus did not form shoot or roots on any treatment tested. Determining the explant tissues that result in the highest frequency of regeneration capacity is an important factor in designing an *in vitro* regeneration protocol. Shoot regeneration in *A. annua* and *A. pallens* was successful in callus derived from leaf and stem explants and leaf and cotyledon explants, respectively. To the best of my knowledge, regeneration of tarragon (*A. dracunculus*) is the only successful example of the regeneration of shoots from callus derived from root explant material in an *Artemisia* species (Ibrahim et al., 2011).

A study on the *in vitro* regeneration in chrysanthemum showed a significantly higher rate in regeneration from petal explants as compared to leaf, stem, and petiole explants (Song et al., 2011). Farroq et al., (2019) tested the regeneration capacity of different rapeseed explant tissue sources. They tested four tissue types (cotyledon, petiole, hypocotyl, and root) for three different regeneration protocols. They found significantly different rates of regeneration depending on the explant source. Interestingly, the explants derived from root tissues did not regenerate on two of the three

protocols and only at low levels on the third protocol. This result supports my observation that callus derived from root explants may not have the capacity to regenerate shoots as easily as callus derived from other tissues.

The observation that root and shoot calli responded differently to the hormone treatments suggests that these calli, even though morphologically similar, differed in their biochemical and molecular characteristics. Genes upregulated and down regulated in auxin-induced callus tissue have been shown to differ in explants derived from root tissue and aerial tissue in *Arabidopsis thaliana* (Fehér, 2019; Xu et al., 2012). Differences in the expression of auxin and cytokinin response pathways in callus derived from various plant tissues in response to wounding have also been reported (Ikeuchi et al., 2013). A study in maize identified several functionally abundant proteins groups in embryogenic calli versus non-embryogenic calli. The groups include proteins involved in energy, metabolism, cell growth/division and secondary metabolism (Varhaníková et al., 2014). Overall, it appears that the totipotency, or ability for callus cells to differentiate into other cell types, is dependent on both genetic elements and many exogenous conditions.

Even though regeneration of shoots and roots from leaf calli was successful, the rate of regeneration was highly variable. One explanation for this variability could be the high heterozygosity of Wyoming big sagebrush. This subspecies is a tetraploid that appears to have originated more than once via hybridization of other *A. tridentata* subspecies and subsequent genome duplication (Huynh et al., 2015). In plants in general, genetic differences within a species have been correlated with differences in *in vitro* regeneration capacity (Ikeuchi et al., 2016). Examples of such differences are common among cultivated crops, where inbreed lines can markedly differ on their ability to

undergo organogenesis in tissue culture (Barandiaran et al., 1999; Cardi et al., 1993; Deglene et al., 1997; Nestares et al., 2002; Zhang & Bhalla, 2004).

The seeds used in this study came from different individuals within the Birds of Prey National Conservation Area. Genetic differences among these individuals, as well as among seeds within individuals, could have contributed to the high variability observed. I attempted to distribute such differences equally among treatments and replicas by randomizing the explants before placing them in the culture medium. However, despite these efforts, the average regeneration potential of the explants within one plate likely varied among plates. Also, by chance, the seeds used in one experiment could have varied in their regeneration capacity from those used in a subsequent replication of the experiment or treatment, thus contributing to the variation between experiments.

The notion that genetic differences may have contributed to variability in regeneration finds support on the results obtained using leaf explants from regenerated shoots. These explants had higher rates of organogenesis than those obtained from seedlings grown from seeds. A possible explanation for these differences is that the first regeneration cycle selected against genotypes with no or low regeneration capacity. Under this scenario, the regenerated plantlets would have come from genotypes with an average regeneration capacity higher than that of some seedlings grown from seeds, thus, leading to higher rates of organogenesis in the former. More clear evidence that seeds from different sagebrush plants produce explants with different regeneration capacity was found in the experiment using half-sibling individuals of the diploid subspecies basin big sagebrush. I cultured these siblings separately, which allowed me to compare the regeneration differences between them. Explants from some of these siblings showed

shoot formation rates of more than 50%. In contrast, in explants from other siblings, shoot formation was minimal or not successful.

Given that individual seeds appear to markedly differ in regeneration capacity, perhaps the most direct approach to obtain consistent and high rates of regeneration would be to select and clone individuals with this characteristic. This selection would be particularly valuable for the subsequent development of genetic transformation protocols such as those using *Agrobacterium tumefaciens* or particle bombardment. These transformation protocols can reduce regeneration due to the selective agents used for selecting the transformed cells (Nofouzi et al., 2019). Consequently, the use of big sagebrush clones with high regeneration capacity is critical to determine whether genetic transformation is feasible in this species (Carvalho et al., 2004; Vogel & Hill, 2008).

Selecting clonal germplasm that has a consistently high regeneration *in vitro* is a common practice in biotech agricultural programs. These clonal lines are used to optimize *in vitro* regeneration and transformation protocols. Moreover, important agronomic traits are often first transformed into these clones, which are then crossed into elite commercial varieties (Glenn et al., 2017). Whether a similar approach could be used to introduce genes in big sagebrush lines that show low *in vitro* regeneration is unclear. Such efforts may be limited by the reproductive cycle of this shrub, which is between two and three years. Thus, the introduction of transgenes in other sagebrush lines may require the development or testing of other *in vitro* regeneration procedures.

An approach to increase the rate and consistency of *in vitro* regeneration would be to test a wider range of media and growing conditions than the ones tested in my study. Many variables in tissue culture can be manipulated to optimize *in vitro* regeneration.

These include the type of basal medium, sugar content, gelling agent, PGRs, light, and temperature (Phillips & Garuda, 2019). A traditional approach for selecting media and growing conditions is to search for successful regeneration protocols in similar species. In genera such as *Artemisia*, information on *in vitro* regeneration protocols, particularly for perennial species, is very limited. Due to this paucity of data, inferring variables to test, other than those I already tested, is difficult. Given these uncertainties, a large array of variables could be used, but this would be very laborious and would require a large amount of plant material with a similar genotype. More recently, Wang et al., (2015) implemented a new approach to select conditions to improve organogenesis *in vitro*. They used information from the transcriptome analysis of hormone response pathways to optimize *in vitro* formation of adventitious branches in seaweed explants. Transcriptome analyses have been conducted in big sagebrush, but thus far, they have focused on changes in terpene synthesis (Huynh et al., 2015). Nevertheless, as more genomic data becomes available, it may provide valuable insights to guide the selection of hormones or other factors that could be used to improve organogenesis *in vitro*.

Apart from obtaining high regeneration rates, the success of an *in vitro* regeneration protocol depends on obtaining plantlets with a normal phenotype (Rojas-Martínez et al., 2010). In Wyoming big sagebrush, the regenerated shoots showed a normal phenotype except when I transferred them to rooting medium containing IAA and NAA and phytagel as a gelling agent. This gelling agent change was necessary to prevent roots from breaking off explants upon removal, however it may have contributed to the development of an abnormal and hyperhydric phenotype.

Hyperhydricity was observed in the basin big sagebrush regeneration treatments. This phenotype occurred in the SIM. At the cell level, hyperhydricity is associated with several changes, including lack of lignin synthesis, highly vacuolated cells, large intercellular spaces, and mesophyll and guard cells with abnormal morphological characteristics (Olmos & Hellín, 1998). Environmental conditions, as well as the PGRs and the gelling agent used in the culture medium, are factors that can contribute to hyperhydricity (Kadota & Niimi, 2003; Mayor et al., 2003; Olmos & Hellín, 1998). These notions are consistent with my observations, the type of hormone used affected hyperhydricity and the type of gelling agent may have also played a role. Solidification of one of the gelling agents used, Phytagel, depends on the formation of bridges through divalent cations such as calcium and magnesium. Uptake of these nutrients by the plant cells may have softened the medium contributing to waterlogging. Ultimately, hyperhydricity is largely attributed to waterlogging, which causes hypoxia, production of reactive oxygen species, and damage to plant cells (Rojas-Martínez et al., 2010).

Many studies have addressed hyperhydricity in tissue culture and ways to mitigate it. Lai et al. (2005) reduced the frequency of hyperhydricity in *Scrophulariaceae* species by periodically venting their culture vessels. Ethylene accumulation in fully sealed vessels can affect plant phenotype (Park et al., 2004). There are commercially available tissue culture vessels with vented/gas permeable lids. The addition of compounds to increase water loss, combat reactive oxygen species (ROS) and antioxidant enzymes in hyperhydric plants has also been reported. The addition of salicylic acid in potato *in vitro* medium reduced the concentration of hydrogen peroxide and antioxidant enzymes activity (Ma et al., 2018). Silver nitrate reduced ethylene accumulation, promoted

stomatal opening and water loss, and reduced ROS in the ornamental plant, China pink (Gao et al., 2017). The addition of silicon to carnation *in vitro* cultures has reduced the frequency of hyperhydricity. Silicon hardens the cell walls in the epidermis and cortex of vascular tissues. It also promotes cell turgidity and normal stomatal opening (Muneer et al., 2017).

For future *in vitro* studies, especially in basin big sagebrush, I would first try to alter the gelling agent and ventilation of vessels to mitigate hyperhydricity. If these methods were not effective, I would begin to experiment with the addition of compounds that have been effective in other *in vitro* systems. While these may be effective at reducing hyperhydricity, there is always a risk that the addition of new compounds may negatively affect regeneration rates.

This study demonstrates that Wyoming big sagebrush can be fully regenerated *in vitro* from leaf explants (**Figure 1.1**). This work also demonstrates that leaf explants from basin big sagebrush can be induced to form callus and shoots *in vitro*. Some sagebrush seedlings or half-siblings had a higher regeneration frequency than others. Assuming that the regeneration capacity is heritable (Nestares et al., 2002), the selection of genotypes with high totipotent callus seems like the most practical approach to optimize the regeneration procedure described in my study. Such optimization would be valuable for genetic transformation as well as the cloning of individuals for genomic analyses and genotypic-plasticity studies.

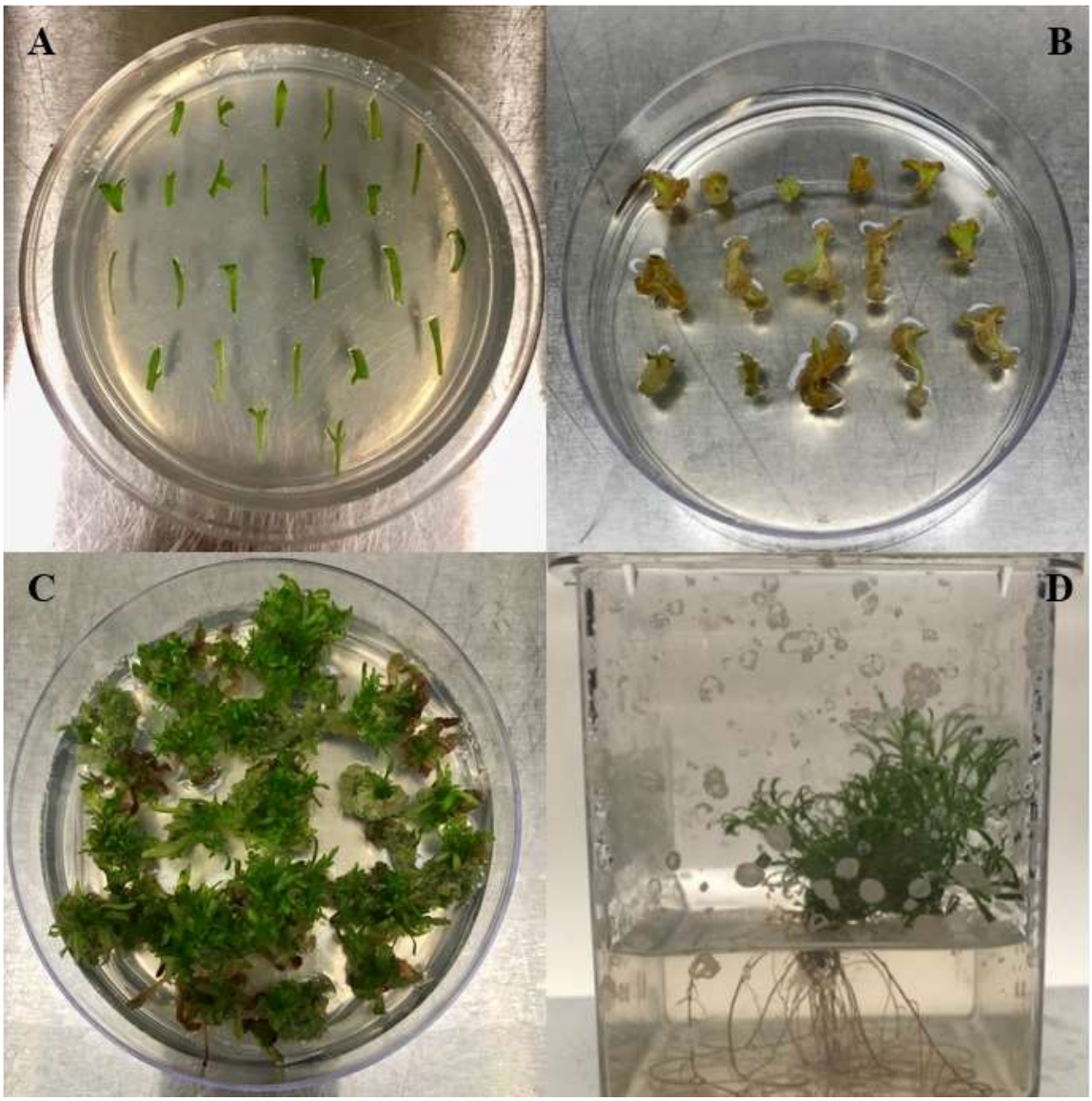

Figure 1.1 ***In vitro*** **regeneration of Wyoming big sagebrush leaf explants. (A) Freshly cut leaf tissue explants. (B) Callus formation on modified MS with 1.0 mg/l NAA and 0.5 mg/l BAP. (C) Shoot formation on modified MS with 1.5 mg/l BAP and 0.5 mg/l NAA. (D) Regenerated shoot with root formation in growth medium.**

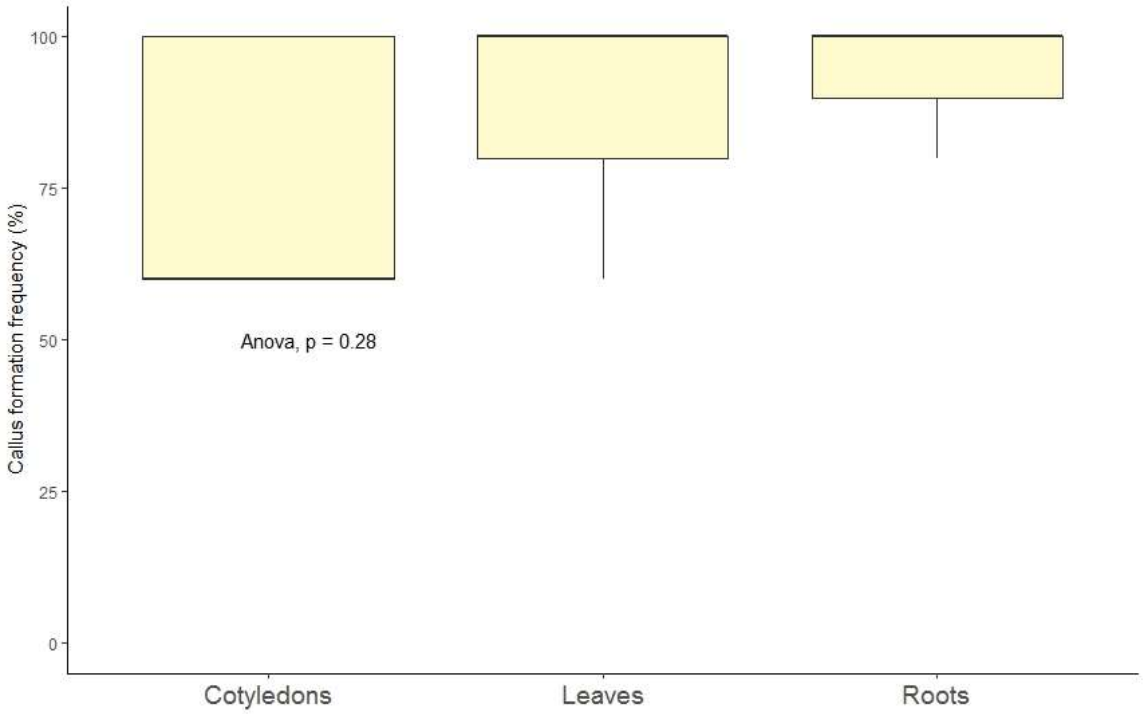

Figure 1.2 Callus formation in cotyledons, leaves, and roots of Wyoming big sagebrush after 4 weeks on modified MS medium containing 1.0 mg/l NAA and 0.5 mg/l BAP

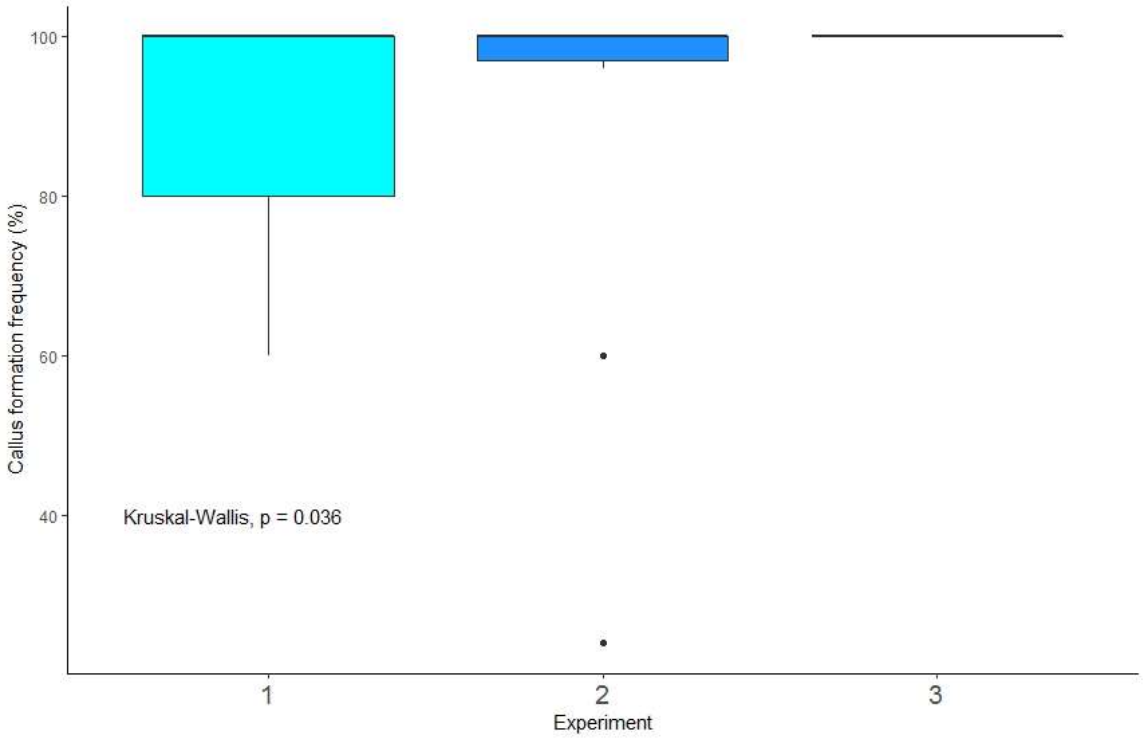

Figure 1.3 Callus formation frequency in Wyoming big sagebrush leaf explants over three experiments on modified MS medium containing 0.5 mg/l BAP and 1.0 mg/l NAA.

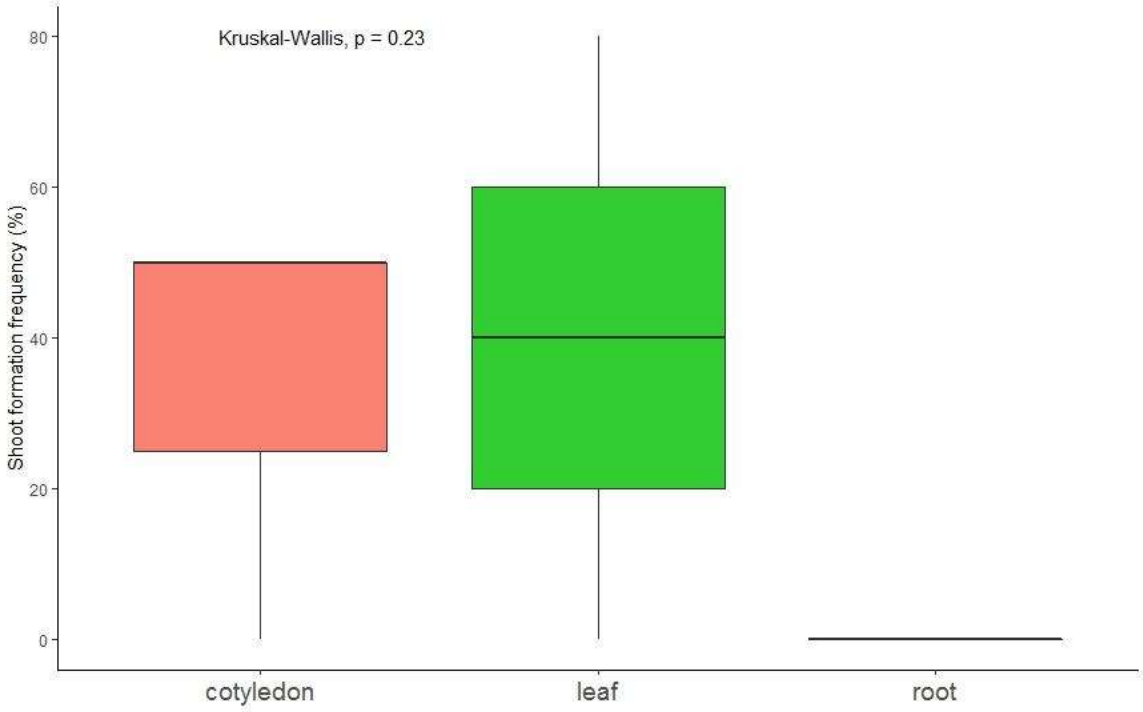

Figure 1.4 Shoot formation frequency in Wyoming big sagebrush by explant type (cotyledons, leaves, and root) on three different PGR containing SIM treatments.

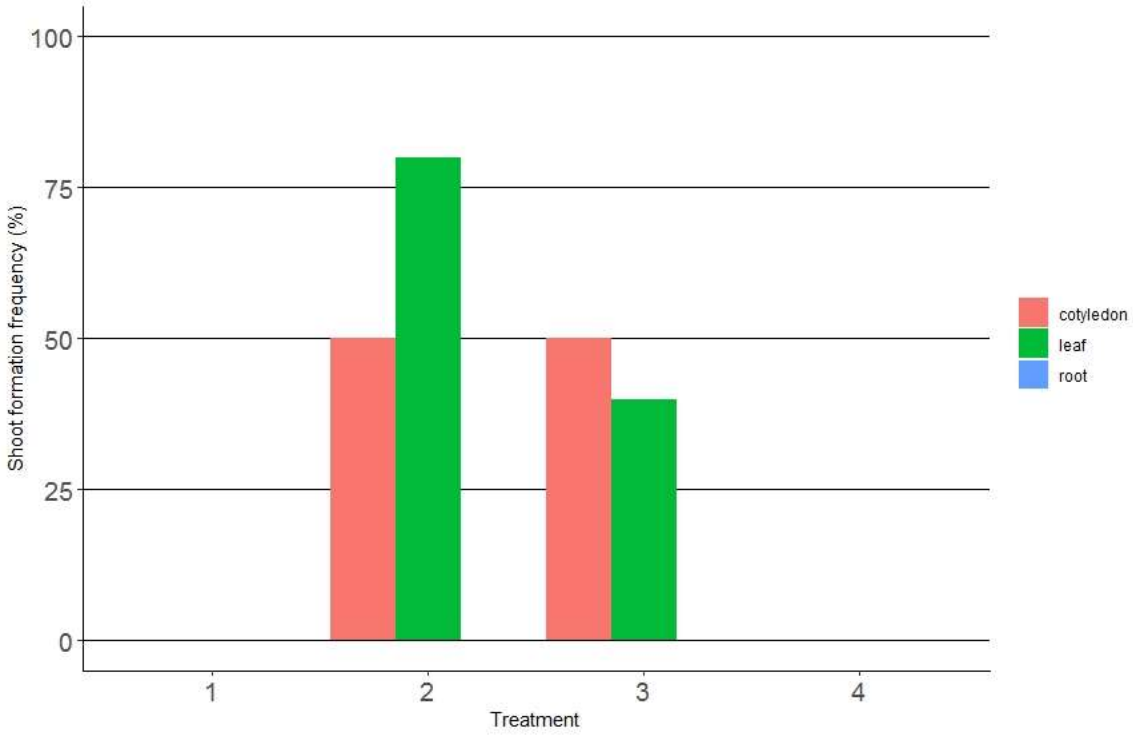

Figure 1.5 Shoot formation of Wyoming big sagebrush leaf, cotyledon, and root derived callus tissue on four treatments containing NAA, BAP and GA_3. Treatment four was the control treatment and did not contain any PGRs.

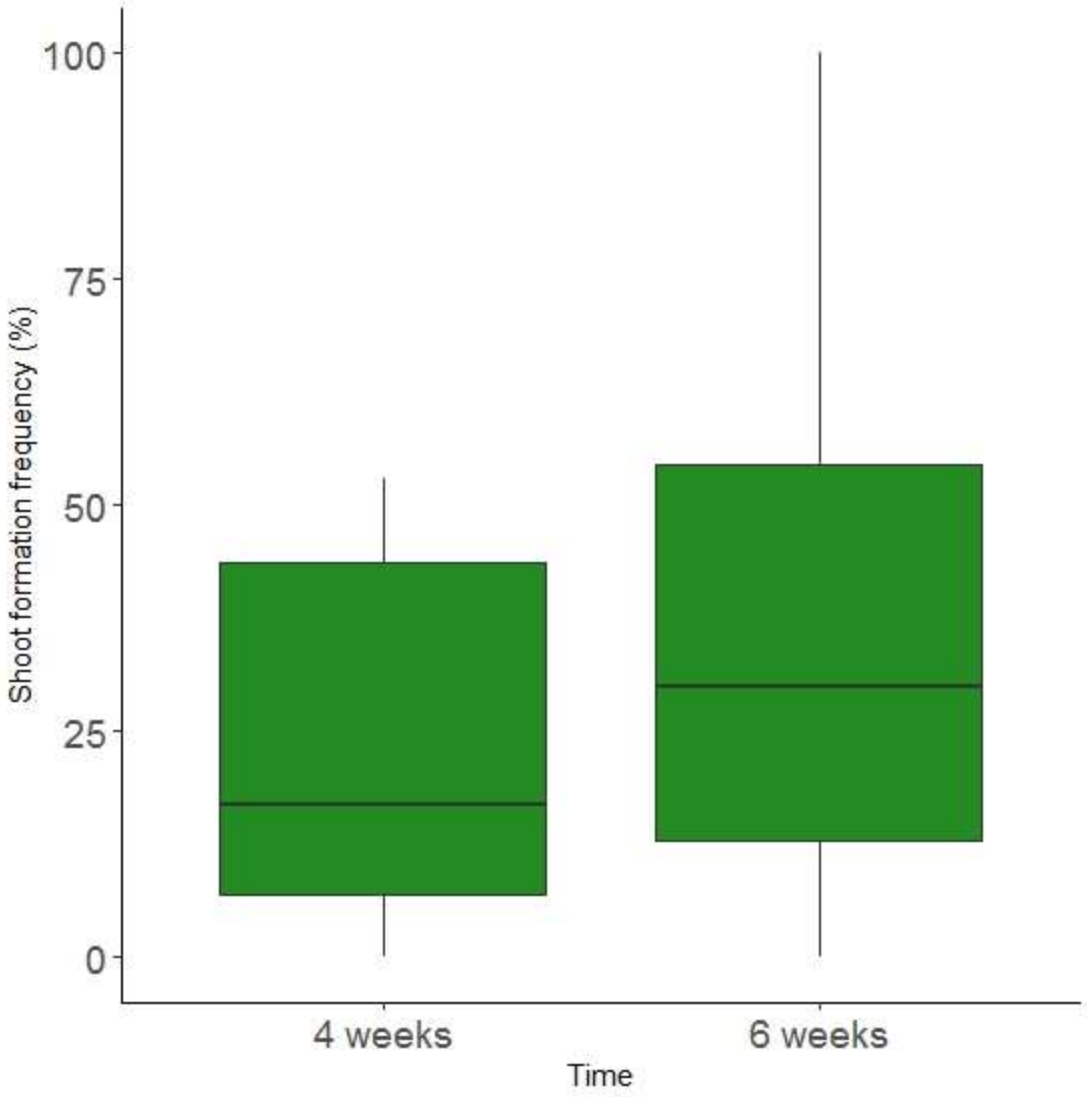

Figure 1.6 Shoot formation frequency of Wyoming big sagebrush leaf explants on modified MS medium containing 1.5 mg/l BAP and 0.5 mg/l NAA after four and six weeks.

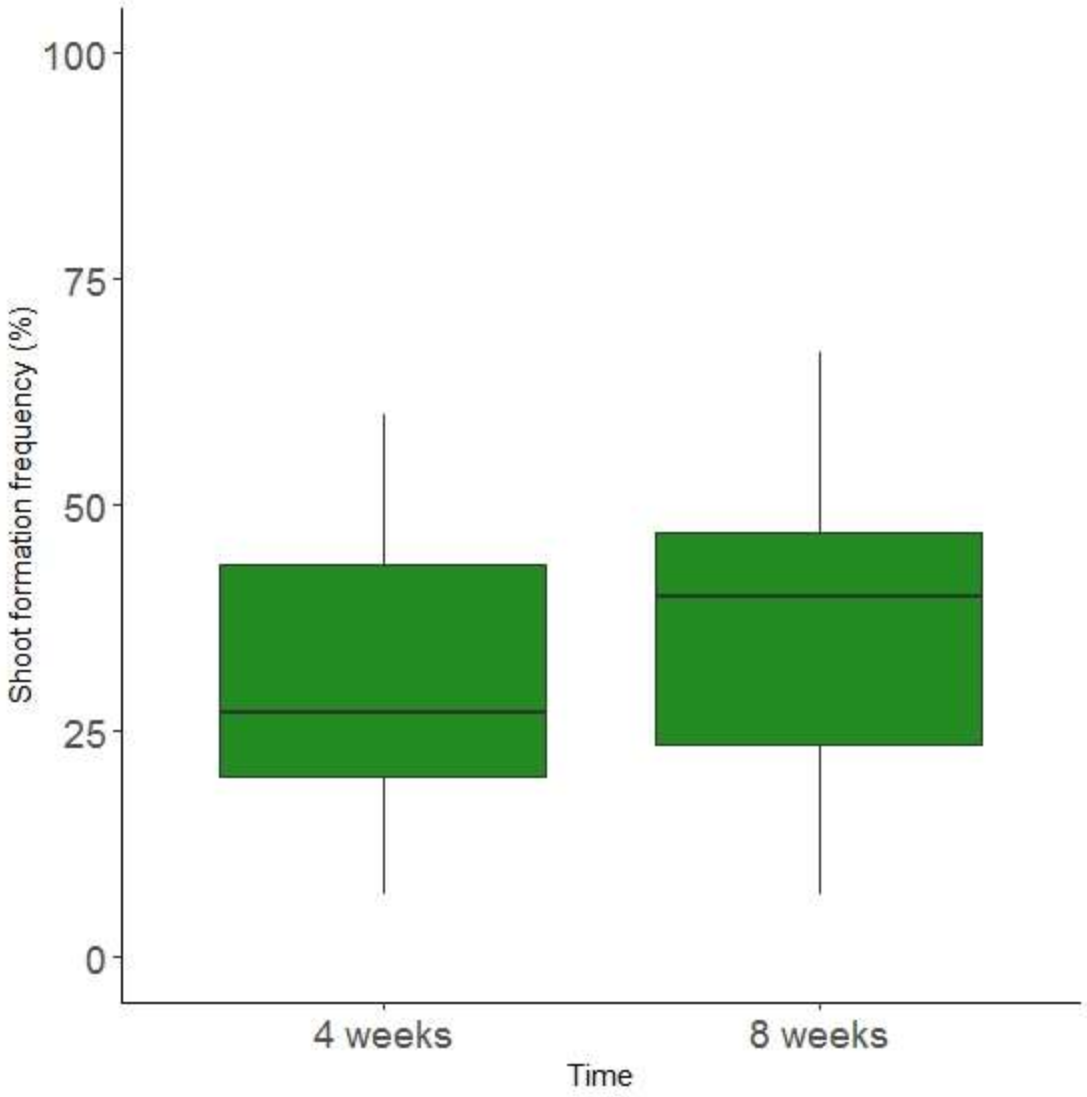

Figure 1.7 Shoot formation frequency of Wyoming big sagebrush leaf explants on modified MS medium containing 1.5 mg/l BAP and 0.5 mg/l NAA after four and eight weeks.

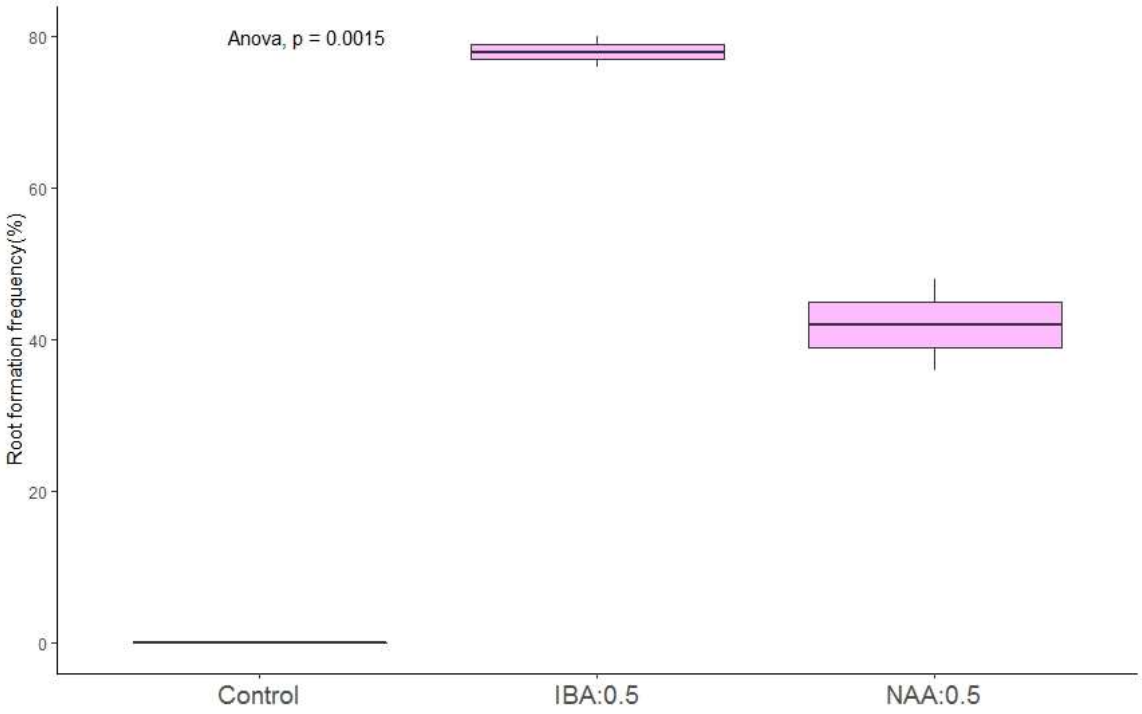

Figure 1.8 Root formation frequency of Wyoming big sagebrush regenerated shoots on modified MS medium containing 0.5 mg/l IBA or 0.5 mg/l NAA.

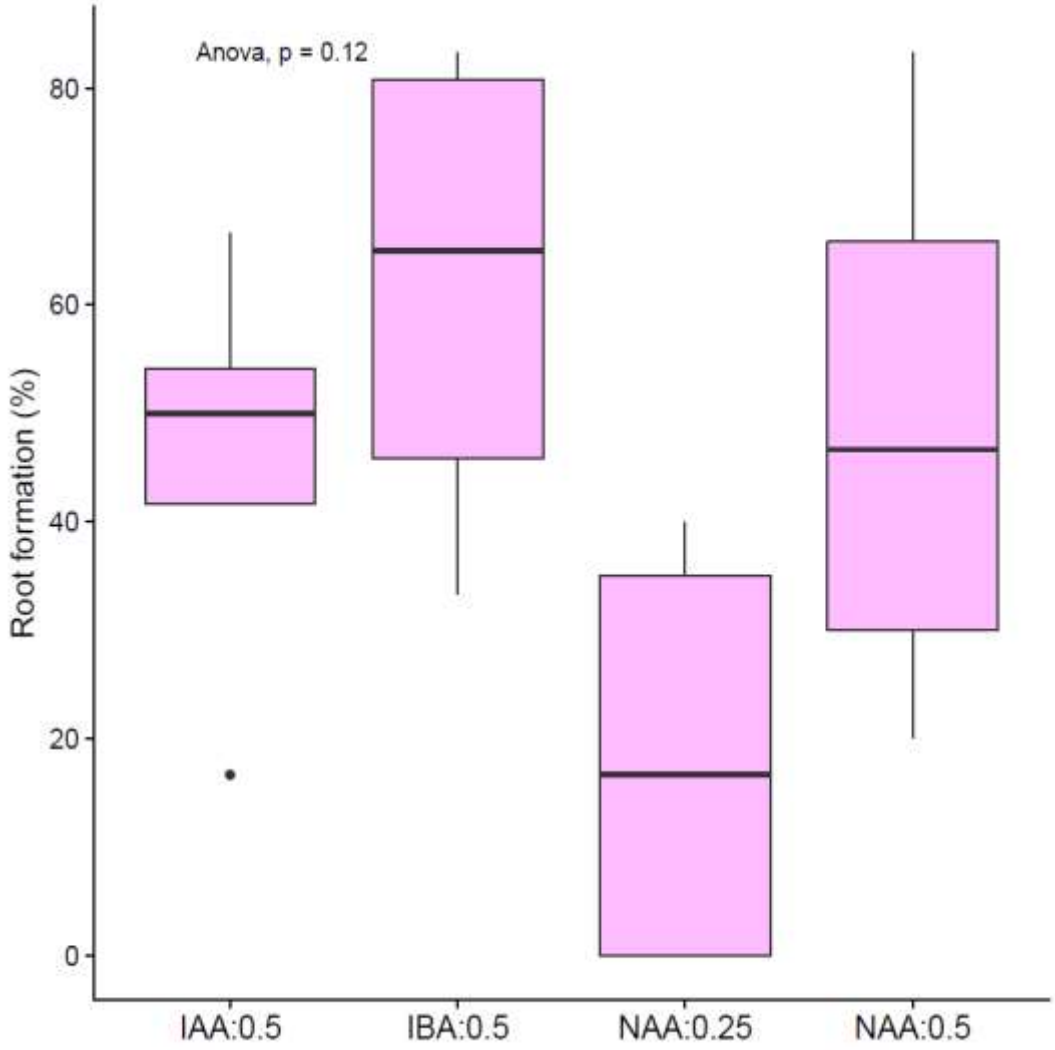

Figure 1.9 Root formation frequency of Wyoming big sagebrush regenerated shoots on modified MS medium containing 0.5 mg/l IAA, 0.5mg/l IBA, 0.5 mg/l NAA, or 0.25 mg/l NAA.

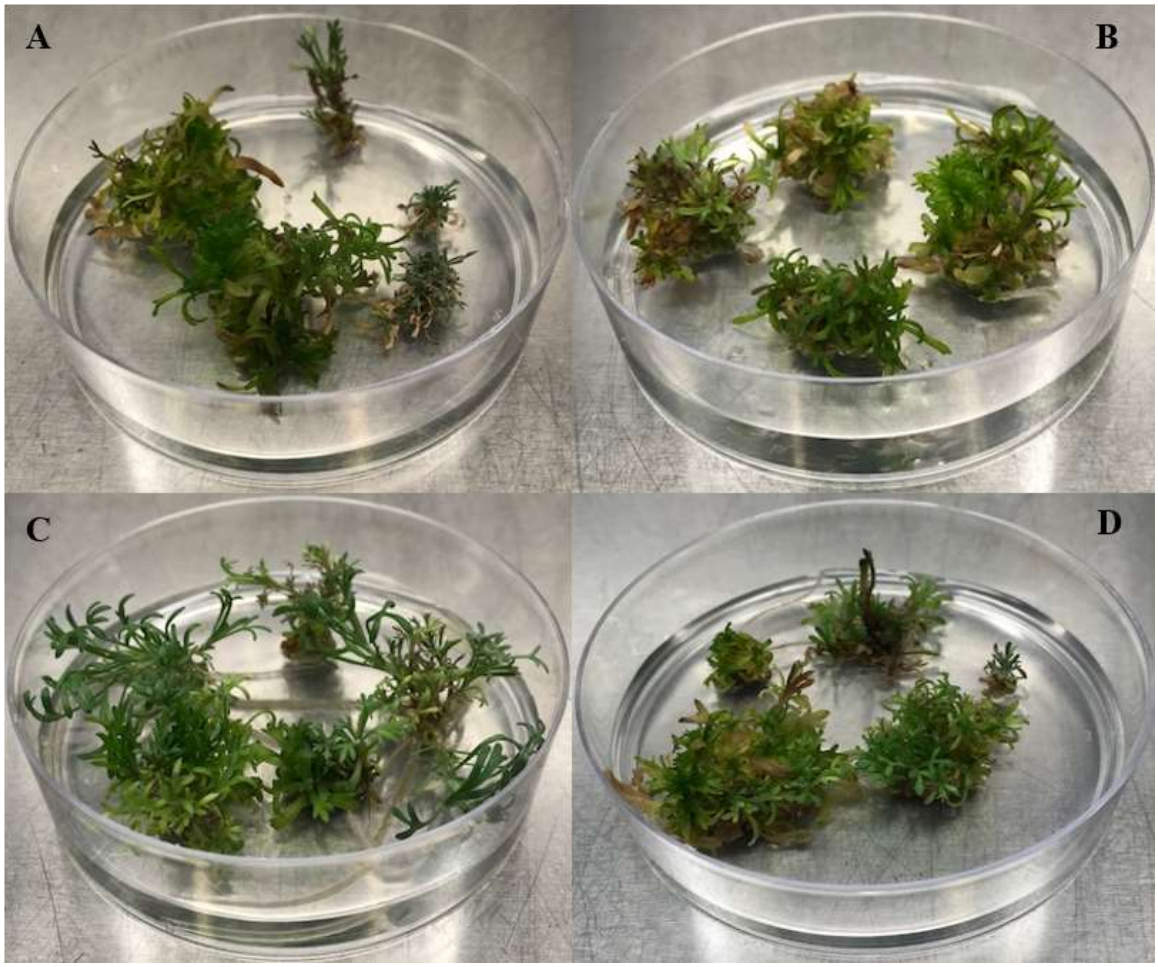

Figure 1.10 Regenerated shoots from leaf explants of Wyoming big sagebrush. Shoots were placed on modified MS medium with the addition of (A) 0.25 mg/l NAA, (B) 0.5 mg/l NAA, (C) 0.5 mg/l IBA, and (D) 0.5 mg/l IAA.

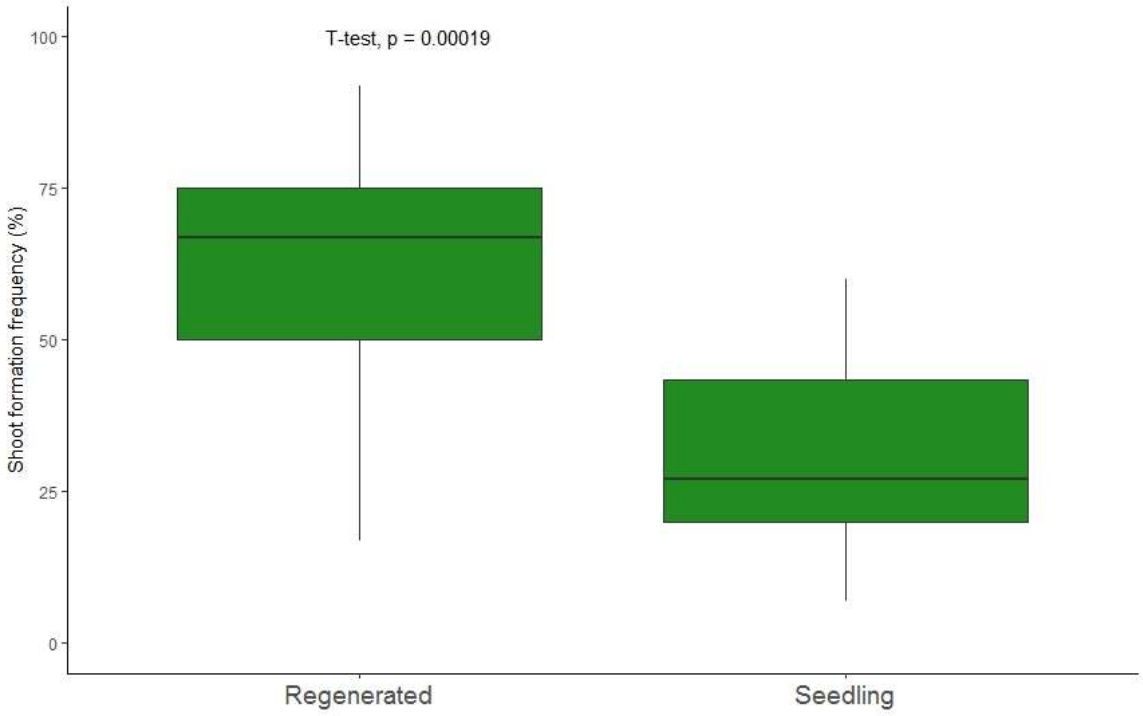

Figure 1.11 Shoot formation frequency of Wyoming big sagebrush leaf explants from regenerated plants and seedlings after four weeks on SIM.

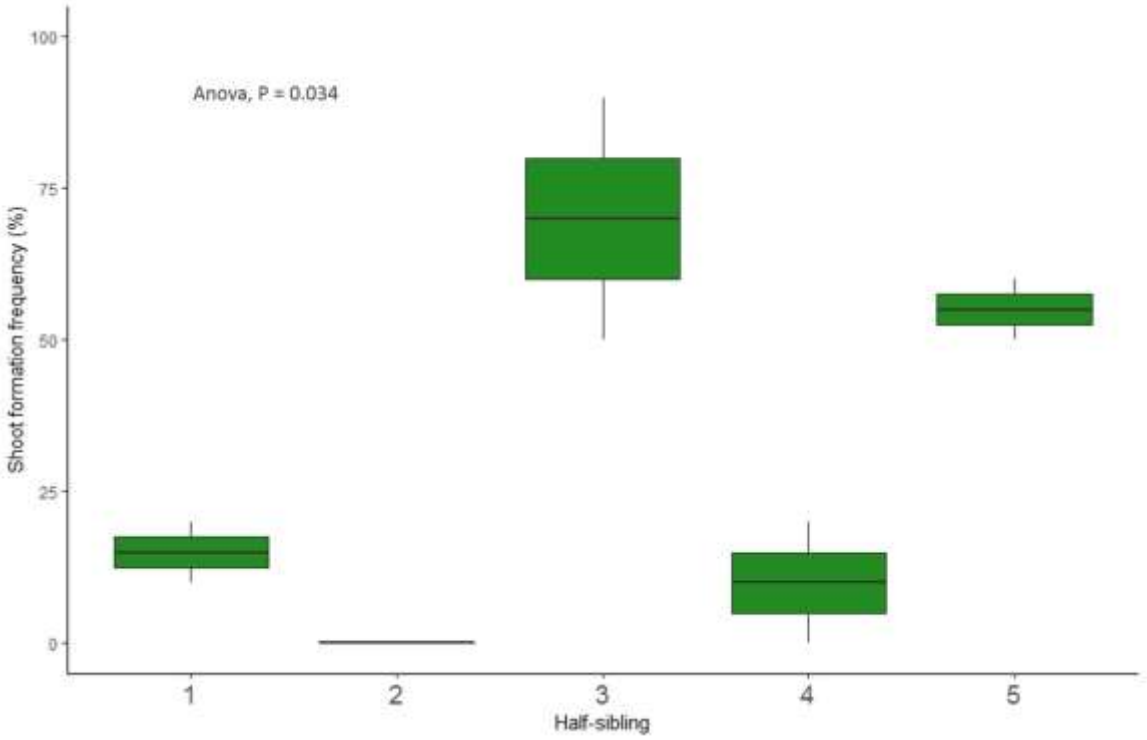

Figure 1.12 Shoot formation frequency in basin big sagebrush leaf explants from individual half-siblings on half or full strength modified MS medium with the addition of 1.5 mg/l BAP and 0.5 mg/l NAA.

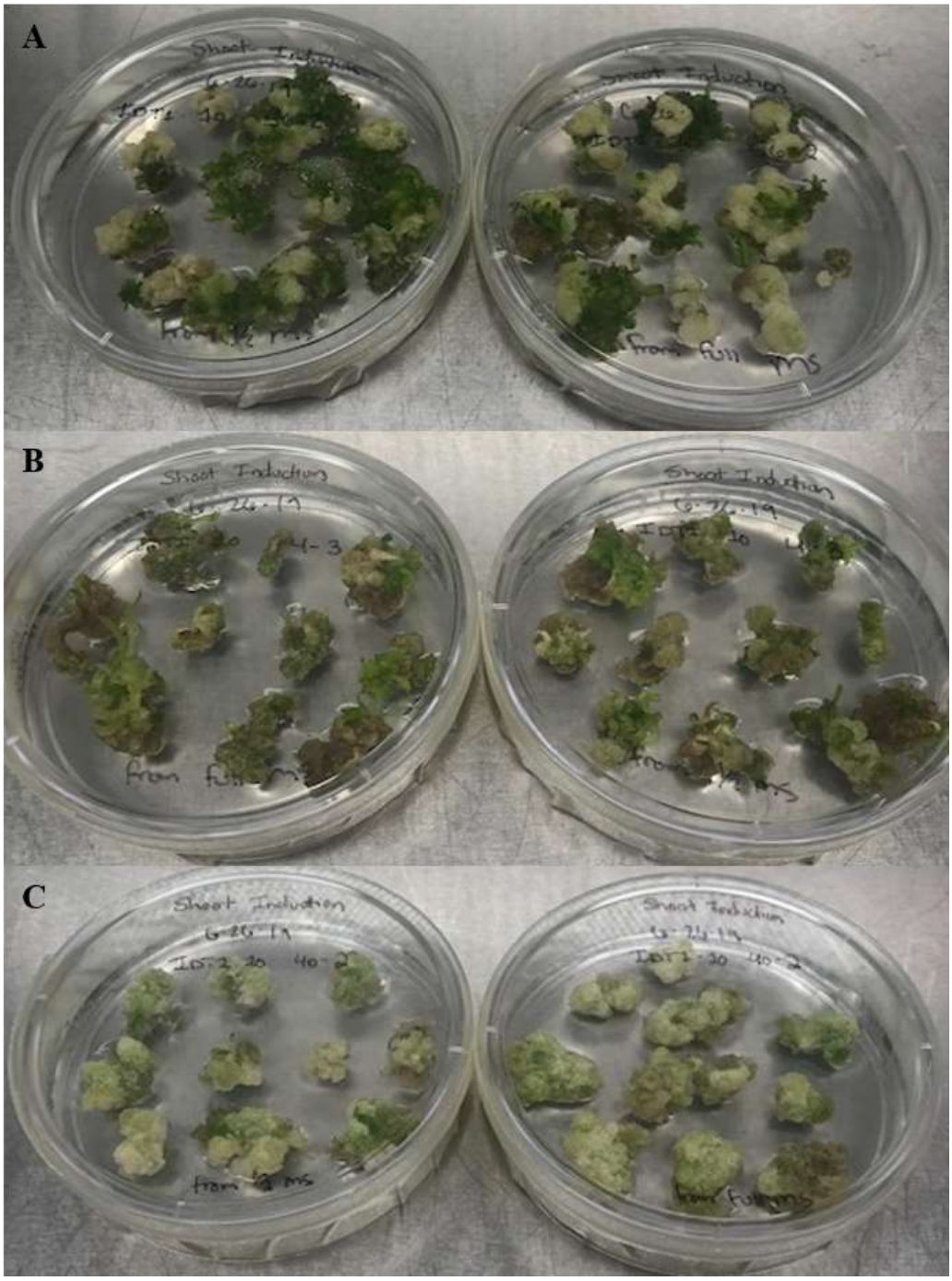

Figure 1.13 Shoot formation in callus derived from leaf explants in basin big sagebrush. (A) Half-sibling with highest rate of shoot formation, (B) Half-sibling with median rate of shoot formation, (C) Half-sibling with no shoot formation.

CHAPTER TWO: *IN VITRO* ROOTING OF *ARTEMISIA TRIDENTATA* SHOOT TIPS AND NODAL CUTTINGS

Introduction

Vegetative propagation, or cloning, is widely utilized in many areas of plant research. While there are some exceptions, clones are genetically identical to the plant they came from. Applications of plant cloning are used in agriculture (Habtamu, 2016), reforestation (Barberini et al., 2015), and ornamental plant production (Preil, 2003). Cloning can be carried out both *in vitro* and *ex vitro*. Different plant explants are used for cloning, but the most common is stem cuttings (Acquaah, 2008; Hartmann et al., 2018) Cloning of this type of explant depends on the ability of the stem to form adventitious roots.

Artemisia tridentata Nutt. (big sagebrush) is a common shrub found in western North America. This plant plays a critical role in sagebrush habitats by increasing soil stability and providing habitat and forage for local animals (Davies et al., 2007; Aldridge & Boyce, 2007). Big sagebrush includes several subspecies that usually occupy different habitats, but on occasions, can overlap and experience hybridization (Kolb & Sperry, 1999; McArthur et al., 1988). Two widely distributed subspecies are basin big sagebrush and Wyoming big sagebrush. Basin big sagebrush often occupies areas with well-drained fertile soils. In contrast, Wyoming big sagebrush is more common in shallow, and rocky soils (McArthur & Plummer, 1978). Both subspecies appear to have a high level of genetic diversity, although this is more pronounced in Wyoming big sagebrush. This

subspecies is a tetraploid that originated more than once through the hybridization of diploid subspecies and subsequent genome duplication (Bajgain et al., 2011; Richardson et al., 2012). These subspecies, and big sagebrush in general, only reproduce sexually and are not known to produce clones naturally (Schlaepfer et al., 2014).

A protocol to obtain clones for each subspecies would provide several benefits. Clones would help to determine the extent to which genotypic or phenotypic plasticity regulates responses to biotic and abiotic factors. Also, the genetic variability within a subspecies, and even a single accession, makes it difficult to conduct experiments with this species. For example, substantial differences in growth rates between individual plants result in high variability within a treatment and complicate the assessment of the effect of an independent variable on the plant. Vegetative propagation of big sagebrush would provide genetically uniform material that would facilitate experimentation. In this regard, cloning would be particularly valuable for genomic and transcriptomic studies. These approaches can give great insights into the responses of plants to environmental stress as well as the molecular mechanisms involved in plant-microbe interactions (Imandi et al., 2015; Schenk et al., 2012). Genomic and transcriptomic techniques depend on the availability of large quantities of genetically uniform material, which cloning can provide (Agarwal et al., 2014). Furthermore, whole-genome sequencing of the cloned material can serve as a reference for the species not only to study function and development but also genome evolution.

Cloning of big sagebrush would also be valuable as an approach to multiply plants with desirable characteristics. For instance, plants that are more drought-tolerant or are more nutritious to threatened animals could be cloned and used for rehabilitation of

degraded habitats. This strategy would require the cloning of selected mature plants. In woody species, *in vitro* cloning of mature plant tissue is, in general, more complicated than cloning from juvenile material. Complications can arise from introducing explants from the field. These explants may have a high load of microorganisms, and thus, it is more challenging to prepare them for the aseptic conditions required *in vitro*. Furthermore, for a variety of reasons, the competence to produce adventitious roots tends to decline with plant age (Benson, 2000; Warrier et al., 2013; Da Costa et al., 2013). Despite these challenges, an attempt to vegetatively propagate adult big sagebrush plants seems a worthwhile endeavor that could be of immediate practical application.

My previous research has shown that clones can be generated through *in vitro* regeneration of leaf explants obtained from big sagebrush seedlings. While this technique of *in vitro* regeneration could be used for a cloning program, it does come with risks to the genetic integrity of the plants. Studies have shown that the regeneration of whole plants from a somatic plant cell can result in phenotypic variation between the clone and "parental" plant. This variation was deemed somaclonal variation by Larkin and Scowcroft in 1981.

Somaclonal variation can be the result of either epigenetic or genetic differences between the clone and parent plant (Schaffer, 1990). Several studies have detected changes in DNA methylation, and histone modifications in plants regenerated *in vitro* (Jaligot et al., 2000; Krishna et al., 2016; Schellenbaum et al., 2008). Although work is still ongoing, somaclonal variation appears to be a result of the artificial environment, and dedifferentiation and redifferentiation of somatic cells (Miguel & Marum, 2011).

The use of certain plant growth regulators (PGRs) also increases the risk of somaclonal variation. Thidiazuron (TDZ.) is a synthetic cytokinin-like PGR that is often used for *in vitro* regeneration in woody and recalcitrant plants. Studies have shown that some concentrations of TDZ used during *in vitro* regeneration cause a variety of undesirable effects, including hyperhydricity, shoot tip necrosis, and somaclonal variation (Dewir et al., 2018). Similarly, the widely used auxin 2,4-dichlorophenoxyacetic acid (2,4-D) has been shown in several studies to cause mutations (Us-Camas et al., 2014).

While an *in vitro* regeneration protocol is necessary for the genetic transformation of most plants, the risk of somaclonal variation can make this approach unsuitable for routine plant propagation. Based on this consideration, I decided to develop a method to propagate big sagebrush from shoot tips and stem cuttings. A previous study reported that bushy seedlings of big sagebrush grown *in vitro* could be split and induced to root (Turi et al., 2014). However, the rate of success of this treatment was not indicated, and the researchers were not able to induce adventitious roots in shoot tips (Turi et al., 2014). A primary goal of my study was to induce rooting in shoot tips and nodal segments. Rooting of these explants would open the possibility of including shoot tip culture in a cloning strategy. Production of many shoot tips via shoot tip culture followed by induction of rooting on them would provide a convenient and practical method to generate a very large number of clones. Apart from mitigating the risk of somaclonal variation, an additional motivation to attempt rooting of shoot tips was to reduce the time required to produce clones. *In vitro* regeneration from leaf explants took approximately 16 weeks; root formation from cuttings was expected to occur in a much shorter time.

Materials and Methods

In Vitro Rooting of Wyoming Big Sagebrush Cuttings

Preliminary Experiment with Shoot Tip and Nodal Cuttings

The Wyoming big sagebrush seeds used in this study were a generous gift from Dr. Joseph Sirotnak (Bureau of Land Management), who collected them from populations of Wyoming big sagebrush within the Morley Nelson Snake River Birds of Prey National Conservation Area in southwestern Idaho.

I isolated seeds and rinsed them under running tap water for approximately two hours. Seeds were then incubated in a 0.5% (v/v) sodium hypochlorite solution containing a surfactant (0.1% TritonX-100) for 10 minutes. Subsequently, the seeds were rinsed four times with sterile water for 5 min per rinse. They were then placed in GA-7 Magenta vessels filled with 100 ml of growth medium. This medium (hereafter referred to as MMS for modified Murashige and Skoog medium) contained ½ strength MS micro and macronutrients (Murashige and Skoog, 1962), ½ strength modified Gamborg (B5) vitamins (Gamborg, 1966), 1% (w/v) sucrose, 3 g/l phytagel and 1 ml/l PPM (Plant Preservative Mixture, Plant Cell Technology). The pH was adjusted to 5.8. After planting, the vessels were kept at ~23°C with a 16 h photoperiod.

With no published data on the *in vitro* rooting of individual Wyoming big sagebrush shoot tips, I first decided to perform an initial cloning experiment using explants from germinated seedlings. From these seedlings, I sampled ten shoot tips and ten nodal cuttings, each containing several nodes, and placed them in Magenta GA-7 vessels containing MMS medium. Shoot tips and nodal cuttings were maintained at ~23°C and exposed to a 16 h photoperiod.

In Vitro Rooting of Nodal Cuttings from Seedlings

For this experiment, nodal cuttings that were approximately 2 cm long were used. After placing these cutting in culture, they had at least one node buried in the media, and one node above the medium. In many cases, the axillary bud on these cuttings had already begun to sprout (**Figure 2.1A**). To induce rooting in these explants, I tested three treatments.

For the first treatment, nodal cuttings were placed on MMS medium containing 1 mg/l NAA and 0.5 mg/l BAP. The idea behind this treatment was to induce callus formation at the cut end in the medium. When callus tissue was formed, at approximately four weeks, cuttings were transferred to RIM consisting of MMS plus 0.5 mg/l NAA. For the second treatment, nodal cuttings were placed directly into MMS medium containing 0.5 mg/l NAA for four weeks. Cuttings that formed roots were transferred to MMS medium containing no PGRs. For the third treatment, nodal cuttings were placed on MMS medium without the addition of PGRs (**Table 2.1**). Each treatment had six replicates with four nodal cuttings per plate. Plates were incubated at ~23°C with a 16 h photoperiod.

Table 2.1 Root Induction in Nodal Cuttings

Treatment	First medium 4 weeks	Second medium 4 weeks
1	MMS + 1 mg/l NAA & 0.5 mg/l BAP	MMS + 0.5 mg/l NAA
2	MMS + 0.5 mg/l NAA	N/A
3 (control)	MMS	N/A

In Vitro Rooting of Sprouted Axillary Shoots from Mature Plants

A similar experiment was conducted to create clones from field-collected material. Branches with new growth were collected from mature Wyoming big sagebrush plants growing at Kuna Butte, Idaho (43° 26’ 47.32” N, 116° 26’ 48.61” W). These branches were wrapped in a moist paper towel and placed in a plastic bag to prevent desiccation. Branches were stored at 4°C and used within three days of collection.

Since this material was collected in the field, I followed a more stringent sterilization protocol. Axillary shoots had started to sprout on the branches, and they were approximately 1-1.5 cm long (**Figure 2.1B**). I removed these shoots using a razor blade, rinsed them under running tap water for about 3 hours, and soaked them in a 70% ethanol solution for 30 seconds. After the ethanol wash, they were moved to a 0.5% (v/v) sodium hypochlorite solution containing a surfactant (0.1% Triton X-100) for 10 minutes. Subsequently, the axillary shoots were rinsed four times with sterile water containing 1 ml/l PPM; each rinse lasted 5 min.

The surface-sterilization procedure likely damaged the cells above the cut end of the sprouted buds. Because these cells are important for the formation of callus or rooting (Ikeuchi et al., 2019), I made a fresh cut at the base before placing the axillary shoots in the medium. Axillary shoots were then placed in the three treatments used in the nodal cutting experiment (**Table 2.1**). Each treatment contained six replicates with ten sprouted axillary shoots per plate. Plates were incubated at ~23°C with a 16 h photoperiod.

Induction of Rooting through Inoculation with *Agrobacterium rhizogenes*

An attempt was made to transform Wyoming big sagebrush shoot tip cuttings with *Agrobacterium rhizogenes*. The protocol for this experiment was adapted from a

transformation protocol for *Medicago truncatula* (Boisson-Dernier et al., 2006). This protocol requires the radicle on a newly germinated seed to be cut and inoculated with a colony of *A. rhizogenes*. Seedlings are then placed on Fahräeus medium. Fahräeus medium is primarily used in symbiotic association studies between legumes and the nitrogen-fixing bacteria *Rhizobium* (Chabaud et al., 2006). The Fahräeus medium has the macronutrients and micronutrients listed in **Table 2.2**, 1.5% agar, and, after adjustment, a pH of 6.5. Following autoclaving, I added $CaCl_2$ to a final concentration of 1 mM.

Table 2.2. Fahräeus Medium Nutrient Composition.

Stock solutions	Stock concentration		Volume	Final concentration
Macronutrients	g/l	M	ml/l	M
NH_4NO_3	40.0	0.5	1.0	0.5 mM
$MGSO_4$, 7 H_20	123.2	0.5	1.0	0.5 mM
KH_2PO	95.3	0.7	1.0	0.7 mM
$NaHPO_4$, $2H_2O$	71.2	0.4	2.0	0.8 mM
Fe-$EDTA_2$	7.3	0.02	2.5	50 µM
Micronutrients				
$MnSO_4$, $CuSO_4$, $ZnSO_4$, H_3BO_3, Na_2MoO_4	1 mg/ml		0.1 each	0.1 µM

The protocol for inoculation with *A. rhizogenes* was modified. Instead of young roots, I inoculated young stems. These stems came from both seedlings that had recently germinated as well as shoot tips from Wyoming big sagebrush seedlings that had been regenerated from leaf explants *in vitro* and grown for approximately 90 days. Cuttings

were inoculated and placed on square 12 cm x 12 cm Petri plates containing Fahräeus medium. Plates containing cuttings without inoculum served as controls. The plates were sealed with parafilm and pierced with several holes to allow airflow. They were then stored at a 45° angle at 23°C with a 16 h photoperiod.

Due to unexpected results observed in my control treatment, I conducted a subsequent test to ascertain my initial findings. From Wyoming big sagebrush seedlings germinated *in vitro* and grown for approximately 90 days, ~2 cm long shoot tips were cut and without inoculation placed on square 12 cm x 12 cm Petri plates containing Fahräeus medium (**Figure 2.3**). Four plates with ten shoot tips each were prepared and incubated under the conditions described above. The root formation frequency was measured at weekly intervals for three weeks.

Albeit with only ten shoot tips, I also tested rooting of shoot tips on MMS medium. Each shoot tip was placed in a 25 x 95 mm culture tube containing 15 ml of the MMS medium. The culture tubes were stored as described above for the plates, and root formation recorded after three weeks.

Cloning of Basin Big Sagebrush

The results with Wyoming big sagebrush indicated that rooting of sagebrush shoot-tip cuttings was feasible. This outcome encouraged me to go one step further and attempt to generate clones of this plant. Dr. Buerki's group at Boise State University is working on *A. tridentata* genomics and was particularly interested in developing a method to clone a diploid subspecies of big sagebrush, basin big sagebrush, to use in their studies. They provided me with seeds from one plant. The seeds from this plant, and the seedlings germinated from them, are referred to as half-siblings. This terminology

reflects that they share the same mother plant, while the pollen may be from another individual. While the primary purpose of this portion of my thesis was to ascertain whether cloning was possible, I also conducted two experiments to address questions that resulted from the rooting experiments with Wyoming big sagebrush.

Seeds were surface sterilized as described earlier and then germinated *in vitro* in the MMS medium. The seedlings were grown in this medium for 140 days before harvesting. This period was approximately 50 days longer than in my previous experiments and led to the sprouting of buds and the formation of numerous small branches in each seedling. The branches collected from one seedling are genetically identical. Consequently, subsequent rooting of these branches would lead to the production of clones and would represent a method to propagate sagebrush asexually. Based on the results with Wyoming big sagebrush, I used the small branches to test rooting in two media and two types of tissue-culture vessels.

Rooting in Fahräeus and MMS medium

For this experiment, MMS or Fahräeus medium was poured into 12 cm x 12 cm square Petri plates. Small branches were collected from 10 half-siblings. The branches were 2-3 cm long and included their shoot tip. Due to the short length of these branches, I will refer to them as shoot tips. From each half-sibling, I collected 12 shoot tips and placed ten of them on top of the Fahräeus medium, and two on the MMS medium. Thus, the shoot tips within each plate were genetically identical and half-siblings of those in most other plates. More shoot tips were placed in the Fahräeus than in the MMS medium due to the limited number of shoot tips available per seedling and based on the notion that rooting would be higher in Fahräeus than in the MMS. The purpose of placing a few

shoot tips on MMS was simply to determine whether rooting would occur in this medium or not. Plates were sealed with parafilm and pierced with several holes. Plates were then incubated at a 45° angle at 23°C with a 16 h photoperiod. After two weeks, I recorded the number of shoots with adventitious roots. Shoot tips that formed roots were then transferred to Magenta GA-7 vessels with MMS medium.

Tissue Culture Vessel: Plates vs. Magenta Vessels

In this experiment, I only used MMS medium, which was poured into Magenta GA-7 vessels and square 12 cm x 12 cm Petri plates. The Magenta GA-7 vessels had 100 ml of the medium, and the square plates had 50 ml of the medium. Seeds were germinated *in vitro* and grown for 140 days. I used five half-siblings and sampled twenty-five shoot tips per half-sibling. One shoot tip was from the main stem, and the others from sprouted buds. Ten tips from each half-sibling were placed with the cut end buried in MMS medium in the vessels. Ten tips from half-sibling were laid on MMS medium in the square plates. Thus, as in the previous experiments, all the shoot tips within a box or plate were genetically identical. Vessels and plates were stored in the same manner as in the last experiment. The number of shoots with root formation was recorded after two weeks.

An additional motivation for the two experiments with basin big sagebrush was to determine the extent that half-siblings vary in their rooting ability. As noted, the shoot tips from one seedling were placed in one plate or box because otherwise, it would have been challenging to keep track of their origin. The shoot tips within a vessel are pseudoreplications. However, the growing environment in plates or vessels with the same medium is nearly identical. Consequently, it seems unlikely that the differences in rooting

ability between half-siblings could be attributed to dissimilarities between replica plates or vessels. Given these considerations, for the experiment using Fahräeus medium, I estimated whether rooting ability varied between half-siblings using pseudoreplications and the Fisher exact test. For the second experiment, this approach was not necessary because the two vessels per half-sibling served as replicates. In this case, the effect of vessel type and half-sibling identity on rooting was analyzed by two-way ANOVA. The data were not transformed before statistical analysis because they satisfied the assumption of normality based on the Shapiro test.

Results

In Vitro Rooting of Wyoming Big Sagebrush Cuttings

Preliminary Experiment with Shoot Tip and Nodal Cuttings

In this preliminary cloning experiment, I found that 10% of the randomized shoot tips formed adventitious roots when placed in vessels containing MMS medium. These shoot tips came from small seedlings; rather than the stem, roots may have formed from the hypocotyl, which could have been part of the cutting. No root formation was observed from the nodal cuttings.

In Vitro Rooting of Nodal Cuttings from Seedlings

Root formation was observed in nodal cuttings that were cut and placed in MMS medium containing 1.0 mg/l NAA and 0.5 mg/l BAP before being moved to MMS containing 0.5 mg/l NAA (**Figure 2.2**). The rate of root formation in this treatment was 12.5% (± 5.6%). Nodal cuttings that were placed in MMS or directly into MMS containing 0.5 mg/l NAA did not show any root formation. I observed that even among the nodal cuttings that did not form roots in the callus induction treatment, the cuttings

enlarged, and the axillary shoot sprouted. A Kruskal-Wallis non-parametric test revealed the difference between these three treatments was significant ($p = 0.033$)

In Vitro Rooting of Sprouted Axillary Buds from Mature Plants

None of the cuttings from the mature tissue collected in the field formed roots. I observed that the material stayed green for approximately two weeks before it started to turn brown and desiccate. Before drying, I observed a small amount of callus on the cuttings that were on MMS containing 1 mg/l NAA and 0.5 mg/l BAP, which is the callus induction medium. The media also started to turn brown indicating leeching of phenolic compounds from the plant material. All treatments were discarded after three weeks.

Induction of Rooting Through Inoculation with *Agrobacterium rhizogenes*

I expected little to no root formation from the shoot tip cuttings on the control plates. Based on the first experiment, only 10% of shoot tips formed adventitious roots in MMS medium that did not contain any PGRs. Much to my surprise, almost 100% of cuttings from regenerated shoot tips formed roots on the control plates (not inoculated and without PGRs). Due to the high rate of root formation in the controls, I did not investigate whether inoculation produced transgenic roots. On the other hand, to ascertain the unexpected results, I repeated the experiment with more shoot tip cuttings using only the control treatment, Fahräeus medium without PGRs and without inoculation. The percent root formation was not as high as in the first experiment with the Fahräeus medium, but root formation was higher than in previous experiments using MMS. For the second experiment using Fahräeus medium, root formation reached a plateau after three weeks at 55.0% (±9.6%) (**Fig. 2.4**).

Cloning of Basin Big Sagebrush

Rooting in Fahräeus and MMS medium

Based on the results obtained with Wyoming big sagebrush on Fahräeus medium, I attempted to generate clones of basin big sagebrush. After harvesting shoot tips from individual seedlings and placing them in Fahräeus medium, the root formation frequency markedly varied between half-siblings (plates) ranging from zero in two half-siblings to as high as 80.0% in another. The average root formation frequency in the shoot tip cuttings of the ten half-siblings was 32% (± 9.3%) (**Figure 2.5**). This average was significantly higher than zero based on a one-sample t-test ($p = 0.007$). Root formation also occurred in shoot tips on the MMS treatment. There were fewer cuttings per half-sibling, and the rate of root formation varied from 0% to 100%. The average total root formation frequency for the ten half-siblings was 35% (± 13.0%). (**Figure 2.6**), which was also significantly higher than zero ($p = 0.025$) and similar to that in the Fahräeus medium.

In Fahräeus medium, ten shoot tips were used per half-sibling. For two of the half-siblings, no root formation occurred. In contrast, in another half-sibling up to eight of the shoot tips formed roots. Although these are pseudoreplications, the results suggest marked differences between half-sibling in their ability to develop adventitious roots. A Fisher exact test using these pseudoreplications indicated a very significant effect of half-sibling identity on rooting ($p < 0.0001$).

Tissue Culture Vessel: Plates vs. Magenta GA-7 Vessels

Root formation was observed in the two types of tissue culture vessels tested. The average rate of root formation for the five half-siblings tested was 32% (± 18.3%) and

28.0 (± 17.1%) in plates and vessels, respectively (**Figure 2.7**). No statistical difference was observed between the two vessels ($p = 0.7$). In contrast, there was a significant effect of half-sibling identity on rooting ($p = 0.016$). Shoots from one half-sibling (55.2) had no root formation in either the plate or box treatment **(Figure 2.8**). In contrast, shoots from another half-sibling (4.3) had a root formation frequency of 90% in the box treatment and 100% on the plate treatment (**Figure 2.9**).

Discussion

The results of this study indicate that shoot tips of big sagebrush collected from seedlings or shoots regenerated from callus can form adventitious roots in media without PGRs. By obtaining several shoot tips from individual seedlings and subsequently rooting them, I was also able to demonstrate the feasibility of cloning big sagebrush *in vitro*.

Rooting of Wyoming Big Sagebrush

Even though I succeeded in rooting Wyoming big sagebrush, there were some inconsistencies between the results of my experiments. In my initial attempts to root shoot tips or nodal cuttings of Wyoming big sagebrush, rooting was minimal in media without PGRs, and only 12% in media supplemented with a synthetic auxin. In contrast, in subsequent tests, rooting in media without PGRs was consistently higher than 50%. Such rooting was first observed unexpectedly in Fahräeus medium for seedlings that served as controls in an experiment aiming at transforming sagebrush using *A. rhizogenes*. The shoot tips used in this experiment had been regenerated *in vitro* from leaf explants. In contrast, the shoot tips for my initial tests were harvested from seedlings germinated *in vitro*. My previous data have shown significantly higher rates of organogenesis from plant material that had already been regenerated (Chapter 1). Thus, I

hypothesized that shoots regenerated from leaf explants might have a higher rooting ability than those from seedlings. However, when I tested the latter on Fahräeus medium, they showed a rather high rate of root formation with an average of 55%. Thus, differences in rooting ability between regenerated shoots and shoot tips from seedlings do not seem to explain the different results entirely.

Another possible explanation for the conflicting results was the different media used in the experiments. The Fahräeus medium differs from the MMS medium that I used in my initial rooting experiments. Such differences include pH and concentrations of mineral nutrients and vitamins. Furthermore, the Fahräeus medium lacked sucrose, while the MMS medium had 1% sucrose. I reasoned that the differences between the two media might have been responsible for the higher frequency of rooting in Fahräeus than MMS medium. However, the test that I conducted in culture tubes with MMS does not support this notion. Eight out of the ten shoot tips placed in these tubes formed roots. Thus, presently I can only speculate about the reasons for the low frequency of rooting in the initial experiment. A possibility is that variations due to maternal effects or genetic makeup result in seedlings that differ in their ability to form roots. Perhaps, by chance, the seeds used in my first experiments yielded a high proportion of seedlings with low rooting ability.

In contrast to young seedlings or regenerated shoots, no root formation was observed in the experiment using mature cuttings. Several explanations could account for the lack of root formation. The more stringent sterilization protocol, containing a 30-second ethanol wash, may have been detrimental to the cuttings. However, callus development in one of the treatments suggests that many cells remained alive. In many

woody species, the transition from juvenile to adult and aging, in general, is associated with a decline in the ability of cuttings to develop adventitious roots (Geiss et al., 2018; Salmi & Hesami, 2016). The molecular events that mediate these changes are complex and include synthesis of inhibitors, differences in the expression of microRNAs, as well as general alterations in signal transduction pathways involved in adventitious root induction (Chen et al., 2020; Gutierrez et al., 2009; Paton et al., 1970; Pizarro & Díaz-Sala, 2019). Given that loss of competence to produce adventitious roots with age is common among woody plants, this may also occur in big sagebrush. Such change would explain the development of adventitious roots in cuttings from seedlings, but their lack in those from adult plants.

The idea that the ability to initiate adventitious roots decreases with aging of big sagebrush needs, however, further testing. The mature cuttings came all from one Wyoming big sagebrush plant in the field. Wyoming big sagebrush is a tetraploid that appears to have originated multiple times through the hybridization of diploid subspecies and subsequent genome duplication (Richardson et al., 2012). This mixed origin has contributed to considerable genetic variation within this subspecies, and it is conceivable that some of this variation also occurs in loci controlling rooting of cuttings. Furthermore, segregation of these loci during sexual reproduction could result in plants with marked differences in their ability to initiate adventitious roots (Shepherd et al., 2008). Thus, it would be valuable to attempt rooting on cuttings for many adult individuals to test whether the lack of rooting that I observed in the mature cuttings reflects a general decrease in the ability to root with aging or a specific characteristic of the individual sampled. Also, it may be worthwhile to experiment with other media and

hormone combinations. While the MS medium is the most widely used in plant tissue-culture (Herman, 2015), others, including, Woody plant medium (WPM) (Lloyd & McCown, 1980), may be useful for micropropagation of big sagebrush. This medium or other changes, such as the addition of charcoal or shading, may reduce the accumulation of phenolics, preventing the browning of the explants and ultimately facilitating adventitious root development (Hildebrandt & Harney, 1988; Thomas & Ravindra, 1997).

Cloning of Basin Big Sagebrush

Growing the seedlings for four months *in vitro* led to the sprouting of many axillary shoots from each seedling. Albeit with some exceptions, the shoot tips collected from individual half-siblings formed roots. Thus, these rooted cuttings represent clones of the seedling from where the shoot tips originated. While these results indicate that big sagebrush can be cloned *in vitro*, some methodological changes are needed to achieve large scale micropropagation. A limitation of the approach that I used is that one seedling only yields a limited number of shoot tips. Shoot tip culture is a well-established technique that could be used to increase the number of shoot tips before rooting (Nehra & Kartha, 1994). To my knowledge, this approach has not been tested in big sagebrush. Yet it has been used successfully to induce multiple shoots from shoot tips and nodes of *Artemisia vulgaris* (Sujatha & Kumari, 2007). The possibility of having many shoot tips per individual seedling would also facilitate the optimization of protocols for rooting and allow better assessment of genotype impact on rooting capacity.

Of the three variables investigated, media, vessel type, and half-sibling identity, only the latter affected rooting. The similarity in rooting frequency between the Fahräeus

and the MMS medium was somewhat surprising, particularly because the former did not have sucrose. Accumulation of soluble sugars at the end of cuttings tends to increase rooting, and sucrose-containing media can enhance sugar buildup (Ahkami et al., 2009; Ruedell et al., 2013). However, before making the shoot tip cuttings, the seedlings were grown in medium with sucrose as well as high concentrations of macro and micronutrients (MMS medium). These conditions may have led to high endogenous levels of soluble sugars in the cuttings, reducing the need for external sugar during adventitious rooting.

Another general factor that can influence rooting is the microenvironment surrounding the cuttings (Chen, 2006; Da Costa et al., 2013). Even though I did not measure specific variables, it seems very likely that the microenvironment in the plates was different from that in the Magenta GA-7 vessels. The ratio of media surface area to vessel volume was higher in the plates than in the vessels. This difference may lead to distinct concentrations of O_2, CO_2, and water vapor in the two types of vessels, which can affect *in vitro* growth (Kozai et al., 1992). Also, the shoot tips were lying on the plates with their cut surface exposed to air. In contrast, the basal end of shoot tips in vessels was inside the medium. Due to this differential placement, the shoot tips in plates could have experienced lower water potentials than those in the vessels, which would tend to benefit rooting in the latter ones (Puri & Thompson, 2003). Despite these probable differences in the microenvironment, the type of vessel used did not have an effect of rooting. This result suggests that, in both vessels, environmental conditions were adequate for rooting.

For various half-siblings, rooting occurred in media without auxin. A high concentration of auxin is a requirement for the induction of adventitious roots, which

usually takes place during the first 96 h following separation from the mother plant (Kevers et al., 1997). This requirement can be fulfilled via polar transport of auxin from the shoot tip and through the application of exogenous auxin. Transport from the shoot tip is sufficient to induce adventitious roots in easy to root species (Da Costa et al., 2013; Steffens & Rasmussen, 2016). The development of adventitious roots in basin big sagebrush without exogenous auxin indicates that at least some half-siblings had a high capacity for rooting. On the other hand, other half-siblings showed little or no adventitious rooting. For these half-siblings, the application of auxin may improve rooting. However, the signal transduction pathways that control adventitious rooting are complex and involve crosstalk with other plant hormones (Gutierrez et al., 2012; Lakehal & Bellini, 2019). Thus, the effect of exogenous auxin on adventitious rooting of basin big sagebrush, and big sagebrush in general, will depend on the specific factors that limited this process.

Independent of the specific factors involved, the apparent variation in adventitious rooting between half-siblings is intriguing. The levels of endogenous auxins, mineral nutrients, carbohydrates, as wells as other metabolites in the mother plants can affect the ability of the cuttings derived from them to form adventitious roots (Druege et al., 2004; Geiss et al., 2018; Ruedell et al., 2013). Like in all plants, the metabolic status of the mother plants is regulated by environmental, developmental, and genetic factors (Da Costa et al., 2013). In my experiment, the mother plants were the half-siblings, which were grown in the same medium and under the same environmental conditions. Thus, it seems unlikely that dissimilarities in light, temperature, or availability of water and nutrients could have led to significant differences in the metabolism and physiology of

the half-siblings and thereby the ability of their shoot tips to form roots. As noted earlier, another factor that could limit adventitious rooting is aging. However, the half-siblings used for the cuttings were the same age, suggesting that differences in their development were not responsible for the observed disparities in rooting competence.

Other possible causes of dissimilarities in adventitious rooting between half-siblings are maternal effects and genetic differences due to cross-pollination and segregation in self-crosses (Donohue, 2009; Karron & Marshall, 1990). Even though the half-siblings came from seeds in the same mother plant, the environment in which they developed could have been different. Within a plant, some seeds begin to form before others and therefore experience different conditions during their growth and maturation (Evans et al., 1991). This situation could have resulted in changes in the nutrients and hormones that the mother plant provided to different seeds. Such maternal effect is known to affect seed germination and seedling growth (Finch-Savage & Leubner-Metzger, 2006). Thus, it is conceivable that maternal effects on germination speed and seedling growth rate gave some half-siblings a competitive advantage over others, and this ultimately affected the levels of endogenous metabolites involved in rooting. Alternatively, the difference in rooting ability between half-siblings may have a genetic basis. Differences in adventitious rooting have been reported for different accessions of the same species (Mishra et al., 2020), but, to my knowledge, not for half-sibling. It would be valuable to ascertain my observations of differences in adventitious rooting between half-siblings. If this is confirmed, determining the role of maternal and genetic effects on these differences would be significant steps toward the identification of factors affecting adventitious rooting.

Overall, the results of this study provide initial information on rooting and cloning of big sagebrush. The data suggest marked differences in adventitious rooting competence between cuttings from mature plants and those from young seedlings. Only the later developed adventitious roots. Variations were also observed in adventitious rooting frequency among cutting from young seedlings. Based on the results with half-siblings, these differences appear to be in part determined by either maternal effects or the genetic makeup of the seedlings. Experiments aimed at investigating these questions will benefit from the implementation of a shoot tip culture protocol. Starting with different seedlings or half-siblings, the production of large numbers of genetically identical shoot tips from each of them would provide sufficient explants to characterize adventitious rooting differences among different genotypes. Furthermore, these explants could be used to test other media and exogenous application of hormones on adventitious rooting. These tests would help to optimize conditions for *in vitro* rooting of big sagebrush shoot tips and ultimately provide an efficient method to clone this plant.

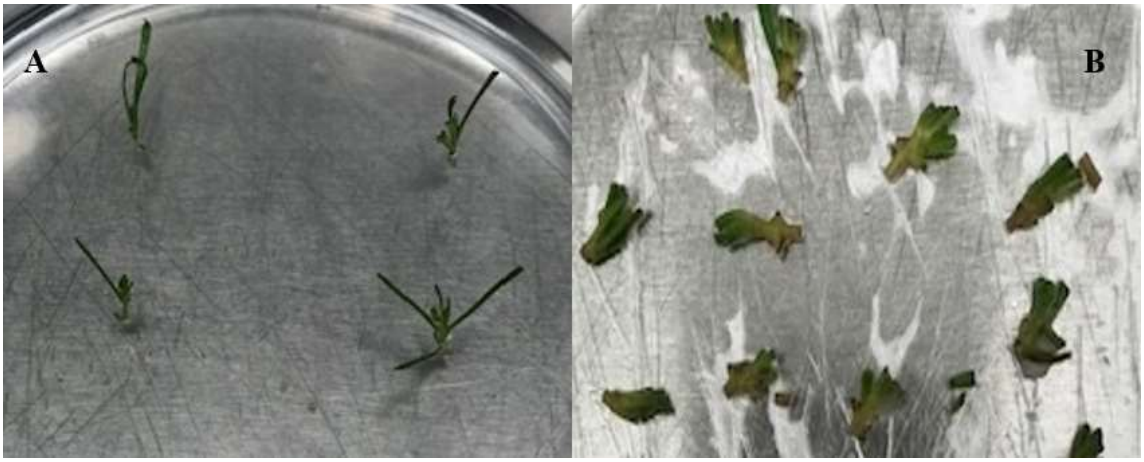

Figure 2.1 A) Nodal cuttings from Wyoming big sagebrush seedlings grown *in vitro*. B) Sprouted axillary shoot cuttings removed from the branch of a field collected Wyoming big sagebrush plant.

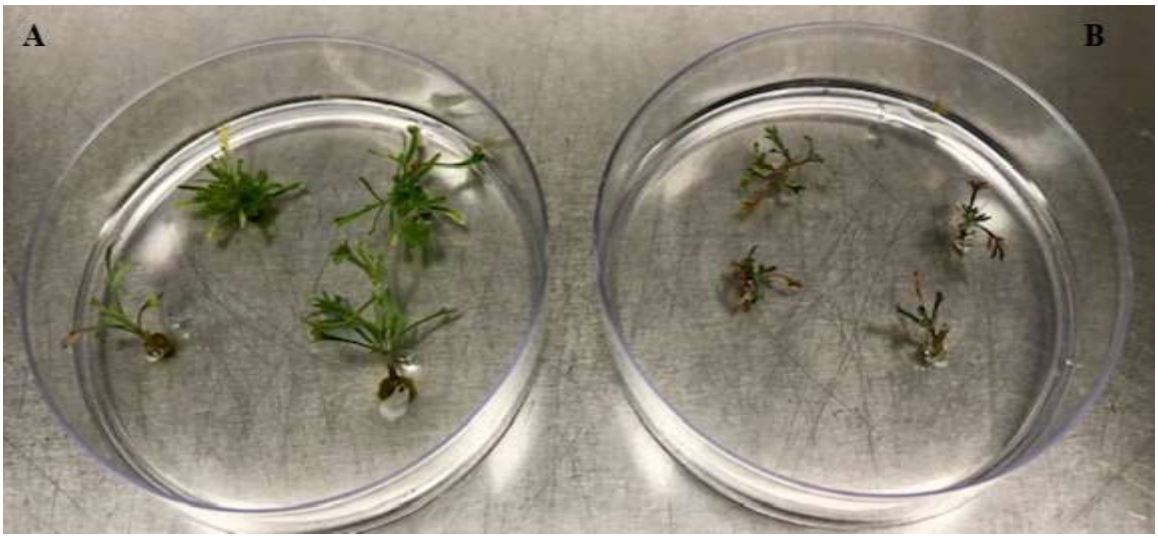

Figure 2.2 Nodal cuttings of Wyoming big sagebrush after four weeks. A) Nodal cuttings on MMS medium containing callus inducing PGRs (1.0 mg/l NAA, 0.5 mg/l BAP). B) Nodal cuttings on MMS medium containing root inducing PGR (0.5 mg/l NAA).

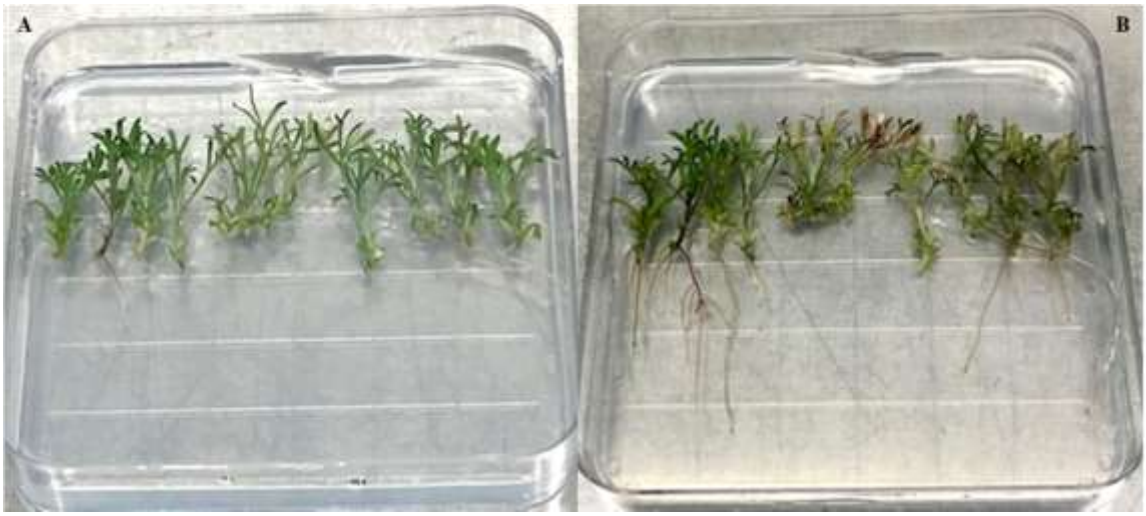

Figure 2.3 Wyoming big sagebrush shoot tips on Fahräeus medium in square 12 cm x 12 cm plates one week (A) and three weeks (B) after being placed on medium.

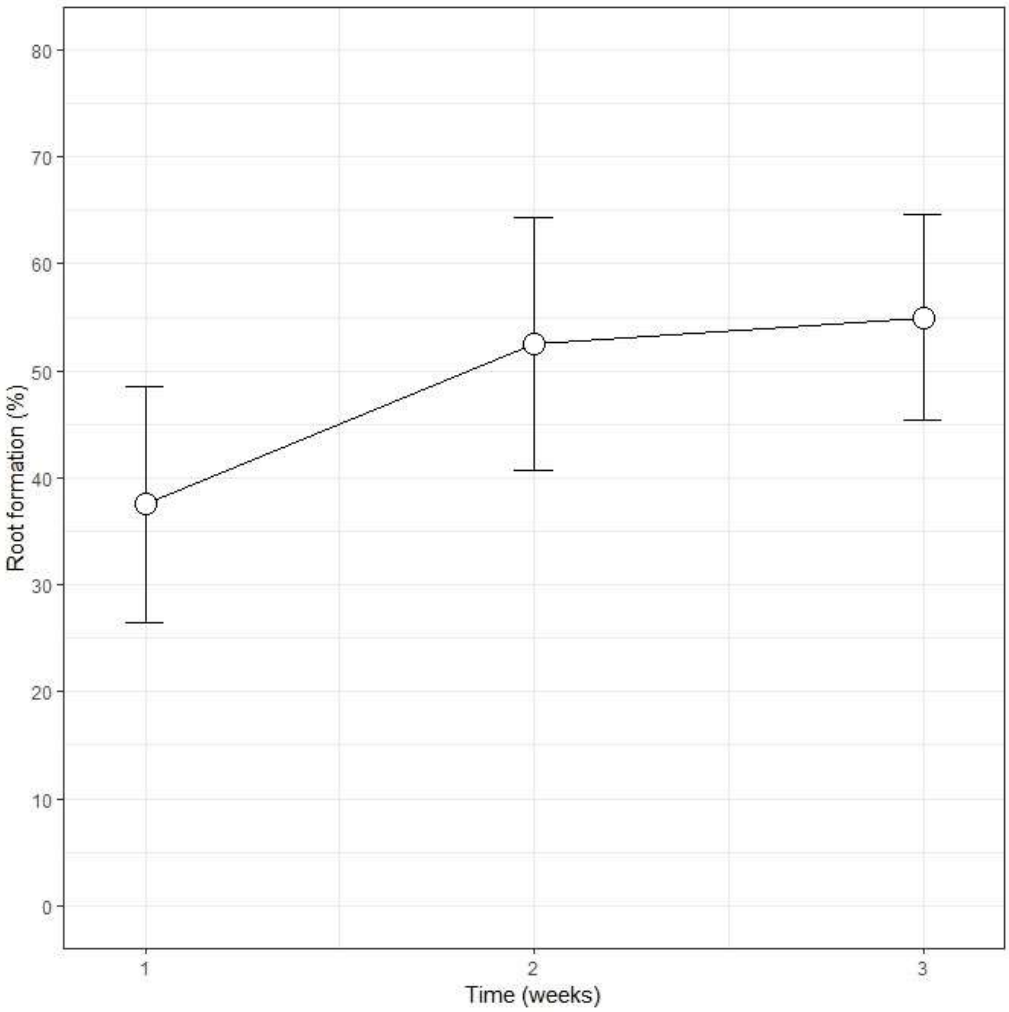

Figure 2.4 Root formation frequency (%) for Wyoming big sagebrush shoot tip cuttings on Fahräeus medium in square 12 cm x 12 cm petri plates.

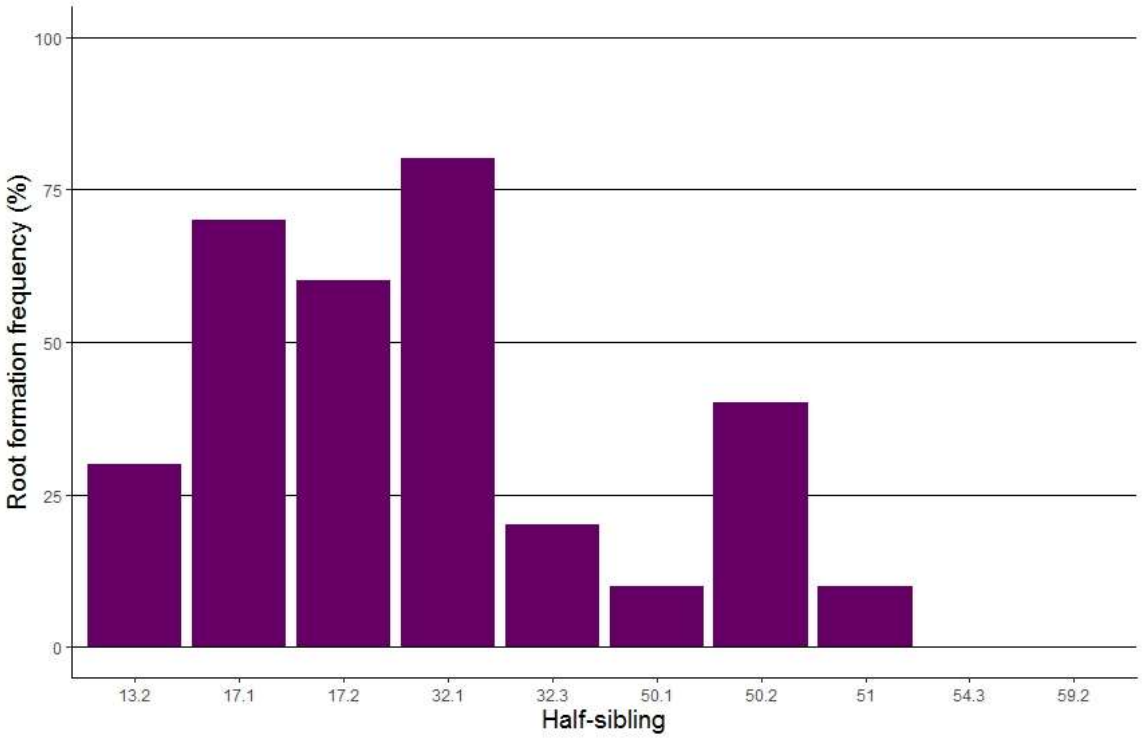

Figure 2.5 Root formation frequency in basin big sagebrush shoot tip cuttings per half-sibling on Fahräeus medium in square 12 cm x 12 cm plates.

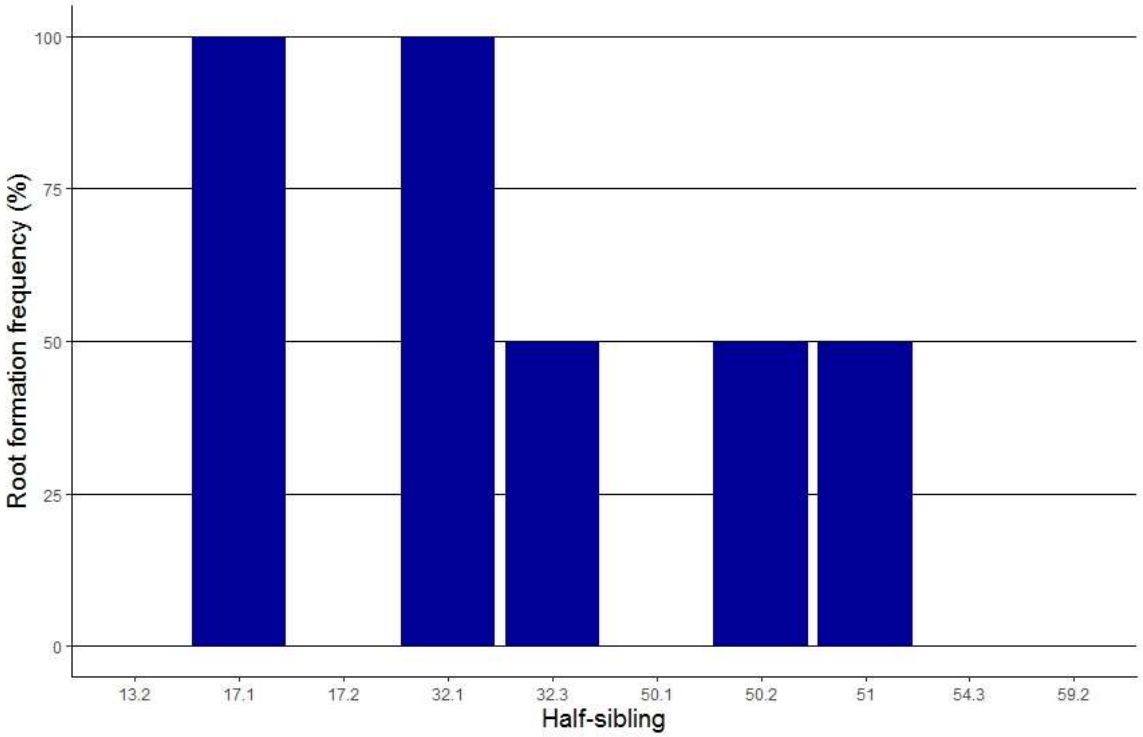

Figure 2.6 Root formation frequency in basin big sagebrush shoot tip cuttings per half-sibling on MMS medium in square 12 cm x 12 cm plates.

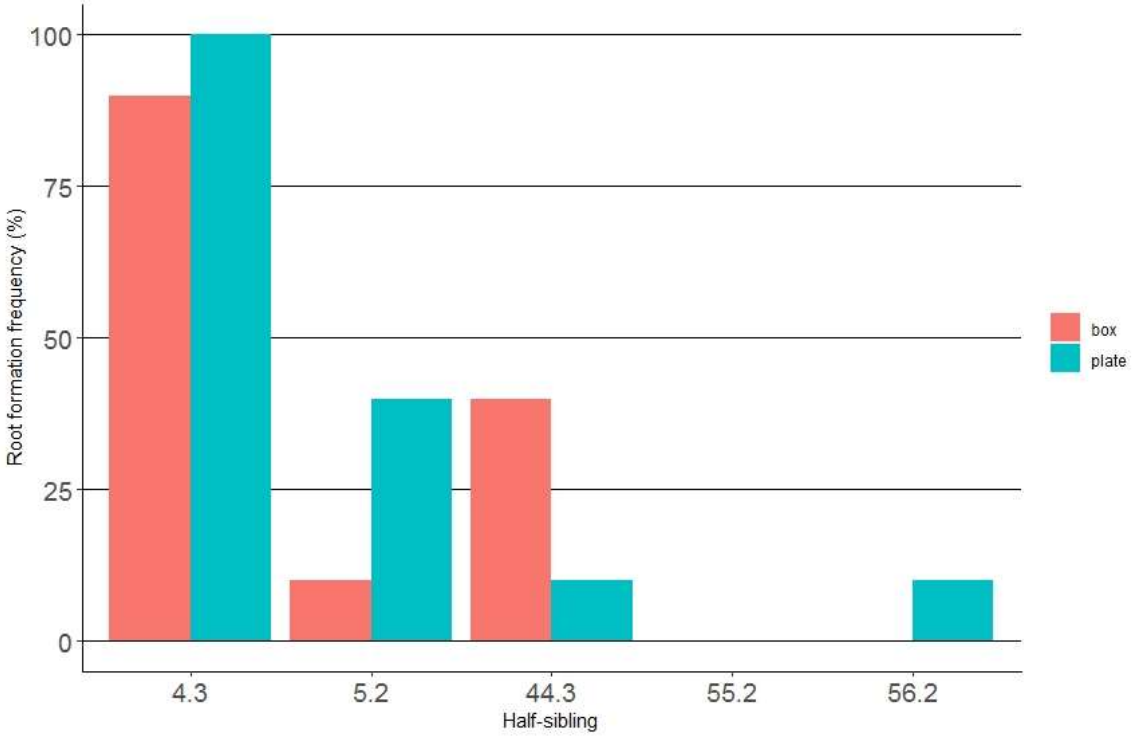

Figure 2.7 Root formation frequency in basin big sagebrush shoot tip cuttings in MMS medium on either 12 cm x 12 cm plates or Magenta GA-7 vessels.

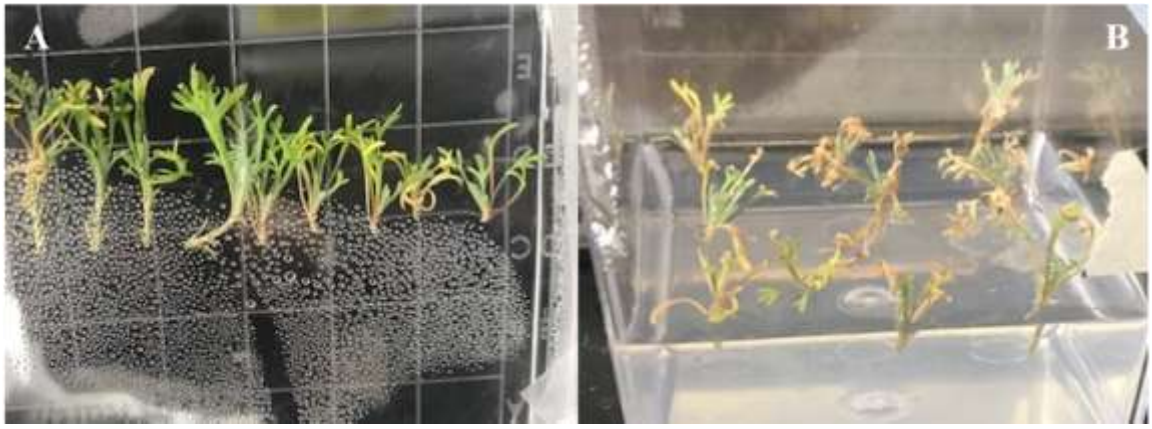

Figure 2.8 Root formation of basin big sagebrush shoot tip cuttings from one half-sibling (55.2) in a square 12 cm x 12 cm plate (A) and Magenta GA-7 vessel (B) containing MMS medium.

Figure 2.9 Root formation of basin big sagebrush shoot tip cuttings from one half-sibling (4.3) in a square 12 cm x 12 cm plate (A) and Magenta GA-7 vessel (B) containing MMS medium.

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