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PROTOCOL NOTE

Applications in Plant Sciences

Acclimation and hardening of a slow-growing woody species emblematic to western North America from in vitro plantlets

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Abstract

Premise: Determining the tolerance of plant populations to climate change requires the development of biotechnological protocols producing genetically identical individuals used for genotype-by-environment experiments. Such protocols are missing for slow-growth, woody plants; to address this gap, this study uses *Artemisia tridentata*, a western North American keystone shrub, as model.

Methods and Results: The production of individual lines is a two-step process: in vitro propagation under aseptic conditions followed by ex vitro acclimation and hardening. Due to aseptic growth conditions, in vitro plantlets exhibit maladapted phenotypes, and this protocol focuses on presenting an approach promoting morphogenesis for slow-growth, woody species. Survival was used as the main criterion determining successful acclimation and hardening. Phenotypic changes were confirmed by inspecting leaf anatomy, and shoot water potential was used to ensure that plantlets were not water stressed.

Conclusions: Although our protocol has lower survival rates (11–41%) compared to protocols developed for herbaceous, fast-growing species, it provides a benchmark for slow-growth, woody species occurring in dry ecosystems.

KEYWORDS

Artemisia tridentata, biotechnology, clonal line, propagation, restoration, sagebrush

Determining the effect of climate change on natural ecosystems requires assessing whether populations possess the ability to adapt, at a sufficient speed, to counteract the effect of climate change (Ofori et al., 2017). Phenotypic plasticity (i.e., ability of individuals to change their phenology, physiology, or morphology without undergoing changes in their genetic makeup) has been suggested as a key component supporting rapid biological responses to climate change by "buying time" for local adaptation to occur, thus ensuring the long-term persistence of populations (e.g., Ofori et al., 2017). Together, phenotypic plasticity and local adaptation are key components determining species' adaptive capacity to tolerate climate change (Dawson et al., 2011), but such knowledge is mostly missing for non-model species. Determining the contribution of phenotypic plasticity requires the development of biotechnological protocols to produce genetically identical individuals (hereafter referred to as individual lines), which are exposed to various treatments reflecting forecasted climatic conditions as part of genotype-by-environment (G×E) experiments. One way to produce individual lines uses individual clones referred to as plantlets (see Murashige, 1974 for a review on this topic) and requires a two-step process: in vitro propagation under aseptic conditions followed by ex vitro acclimation and hardening to prepare plantlets for G×E experiments. Due to aseptic growth conditions, in vitro plantlets exhibit abnormal phenotypes (e.g., little epicuticular wax deposition, stomatal abnormalities such as poorly functioning guard cells, and deficient root hydraulic conductivity; Fila et al., 1998; Hazarika et al., 2006; Chandra et al., 2010), which are maladapted to ex situ conditions. For instance, rapid desiccation usually occurs when in vitro plantlets are exposed to laboratory conditions where humidity levels

are much lower (i.e., 23%) than the >98% humidity level they were cultured in (Hazarika et al., 2006). Therefore, in vitro plantlets must go through an acclimation and hardening process to develop functional leaves and rooting systems (Fila et al., 1998; Hazarika et al., 2006; Chandra et al., 2010). Such morphogenesis has been more easily applied to herbaceous, fast-growing, annual plants (e.g., tomato, sunflower; Cruz-Mendívil et al., 2011; Nowakowska et al., 2020), but is challenging with woody, slow-growing, perennial species (Oakes et al., 2020). Indeed, most of these species share similar anatomical characteristics (e.g., amphistomatous leaves, clonal splitting; Downs and Black, 1999; Schenk et al., 2008; Drake et al., 2019) preventing the application of classical biotechnological protocols. This publication aims at filling this gap of knowledge by focusing on the keystone slow-growing shrub, *Artemisia tridentata* Nutt. (Asteraceae), endemic to the imperiled western North American sagebrush steppes (McArthur et al., 1981; Remington et al., 2021). This protocol would also contribute to the ex situ conservation of threatened taxa and advance fundamental biological knowledge by facilitating the investigation of the molecular mechanisms underpinning phenotypes.

Within the genus *Artemisia* L., in vitro and ex vitro protocols are available for herbaceous species (mostly focusing on pharmaceutical species such as *A. annua* L.). In herbaceous *Artemisia* species, acclimation and hardening of in vitro plantlets to ex vitro conditions can be achieved easily due to the fast setting of functional leaves during the first stages of acclimation, leading to high survival rates (e.g., 65–80% in *A. annua*; Hailu et al., 2013; Zayova et al., 2018). Although approaches to acclimation are similar across studies, soil mixes and duration of plantlets' exposure to high humidity differ (Hailu et al., 2013; Zayova et al., 2018), but these differences do not seem to impact survival. On the other hand, a lack of consistency in slow-growing, woody species has been shown to have repercussions on survival (Oakes et al., 2020), necessitating standardized protocols for these taxa. Developing and validating such protocols to slow-growing, woody *Artemisia* species has not been achieved yet, but an in vitro propagation method is available for *A. tridentata* (Barron et al., 2020), which provides the starting material for the protocol presented here.

The protocol presented here promotes structural and physiological changes of in vitro–grown *A. tridentata* plantlets, resulting in functional ex vitro "seedling-like" plantlets (Figure 1). We anticipate that this protocol will serve as a benchmark for slow-growing, woody species occurring in dry ecosystems worldwide. We describe and demonstrate the efficacy of our protocol here; the detailed, step-by-step protocol and materials list are available at protocols.io (doi.org/10.17504/protocols.io.j8nlk4zpxg5r/v2; Martinez et al., 2022). The overall protocol is subdivided into two major steps: (i) in vitro to ex vitro plantlet acclimation under controlled laboratory conditions (16 weeks), and (ii) preparing acclimated plantlets for G×E experiments (plantlets are usually kept in a greenhouse setting up to the age of six months). In line with similar studies (Hazarika et al., 2006; Chandra et al., 2010), survival was used as our main criterion to determine successful acclimation and hardening of in vitro–grown plantlets to ex vitro conditions; we differentiate between survivorships associated with the two major steps (Tables 1 and 2). Because survival depends heavily on leaf morphogenesis, we determined successful phenotypic changes by inspecting leaf anatomy (both macro- and micro-morphologies). Shoot water potential (Ψ_{shoot}) was used as a metric to ensure that plantlets were not water stressed prior to starting G×E experiments. To maximize survival, soil water potential (Ψ_{soil}) was used to guide the watering regime during in vitro to ex vitro acclimation and hardening.

METHODS AND RESULTS

Plant material and protocol validation

This study focuses on diploid ($2n = 2x = 18$) *A. tridentata* (hereafter referred to as sagebrush), which has been shown

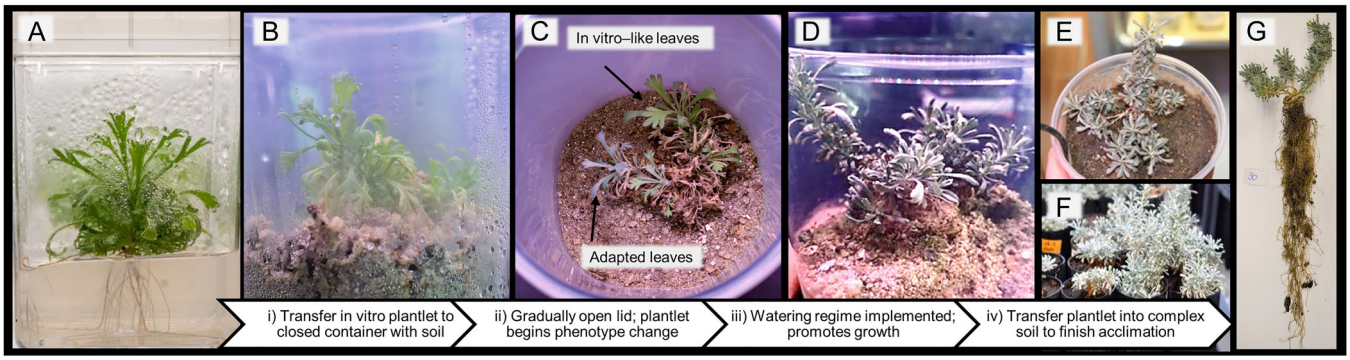

FIGURE 1 Summary of the steps from in vitro–grown diploid *Artemisia tridentata* plantlets to ex vitro conditions. At the end of the acclimation and hardening process, the plantlets exhibit similar phenotypes as sagebrush seedlings. (A) In vitro–grown plantlet. (B) Transplanted plantlet from in vitro to sandy soil in a lidded vessel. (C) Plantlet undergoing hardening process. (D) Opened vessel with watering regime implemented. (E) Transplanted plantlet into silty soil. (F) Plantlets in greenhouse conditions. (G) Acclimated and hardened plantlet with root structure.

to exhibit a faster growth rate compared to its polyploid counterpart, making it a better model for this protocol (Richardson et al., 2021). We used two in vitro individual lines of sagebrush representing two genotypes (Barron et al., 2020) maintained in culture at Boise State University to develop and validate our protocol. The G1 individual line originated from Idaho, USA (43°20′09.6″N, 116°57′50.4″W, 1304 m a.s.l.) and the G2 individual line originated from Utah, USA (38°18′21.6″N, 109°23′13.2″W, 1825 m a.s.l.). Successful phenotype change was determined by inspecting leaf anatomy and indumenta, as well as stomatal anatomy (i.e., epidermal leaf peeling), using a Leica EZ4 dissecting microscope and a Leica CME light microscope (Leica Microsystems, North Deerfield, Illinois, USA) (Downs and Black, 1999) (Figures 1 and 2). Data for assessing qualitative phenotypic change were generated by using the *Arabidopsis thaliana* (L.) Heynh. stomata tape-peel protocol (only focusing on step 2; Lawrence et al., 2018) with the following modifications: (i) in the case of ex vitro plantlets, trichomes were removed using the back of a spatula prior to using the Scotch tape (step 2.2) and (ii) peels were bathed in distilled water instead of the stomata opening buffer (step 2.4) prior to mounting for microscopy. Preliminary data from our lab showed phenotypic variation in leaf traits across sagebrush populations; however, in general within a sagebrush individual line, in vitro plantlets differ from ex vitro plantlets by (i) having dissected leaves, (ii) lacking trichomes, (iii) exhibiting nonfunctional guard cells, (iv) larger stomata, and (v) lower stomatal density (Figure 2). Finally, it is worth mentioning that no phenotypic differences were observed between the abaxial and adaxial sides of the leaf (i.e., sagebrush has amphistomatous leaves; Downs and Black, 1999).

Ψ_{soil} was used to guide the watering regime (using TEROS-21 probes; METER Group, Pullman, Washington, USA) during in vitro to ex vitro acclimation and hardening to reduce mortality at this critical step of our protocol. Ψ_{soil} should be maintained above −0.1 MPa for well-watered conditions and between −0.2 and −0.9 MPa for hardening purposes (Drivas and Everett, 1988; see Figure 8 in Martinez et al., 2022). In addition, we found that when the plantlets in silty soil (see below for more details) were exposed to soil water potentials of −1 MPa (see Figure 8 in Martinez et al., 2022), the Ψ_{shoot} declined to about −2.2 MPa, which based on preliminary work in our lab, could lead to stomatal closure in sagebrush seedlings and mortality by hydraulic failure. Thus, the watering regime recommended in our protocol is more conducive to optimal growth in preparation for G×E experiments. Finally, Ψ_{shoot} is used to confirm that plantlets are well-watered prior to conducting G×E experiments (Table 2). Our preliminary work showed that well-watered plantlets in the greenhouse had an average Ψ_{shoot} of −0.89 MPa (G1) and −1.58 MPa (G2) (Table 2). This Ψ_{shoot} is considered low for species from mesic habitats, but it is common for sagebrush plants under moist spring conditions when growth is maximum (Kolb and Sperry, 1999; DiChristina and Germino, 2006). The section below summarizes the in vitro to ex vitro protocol and preparations for the G×E experiments.

In vitro to ex vitro protocol

Preliminary analyses revealed that in vitro plantlets should be between 11 and 16 weeks old to be suitable for this protocol. However, no differences in survival have been observed between 11- or 16-week-old plantlets, suggesting that users can successfully apply this protocol within this time period (Table 1, Appendix S1). The protocol applied to produce ex vitro–acclimated sagebrush plantlets consists of the following four major sub-steps and takes 16 weeks to complete: (i) transfer of in vitro plantlets from modified Murashige and Skoog medium (Barron et al., 2020) to a sand–vermiculite mix (4:1 v/v; hereafter referred to as sandy mix) in a lidded vessel to initiate plantlet establishment and root growth under high (99%) humidity (four weeks); (ii) gradually opening the vessels to decrease humidity (33–45%) and increase gas exchange, which induces

TABLE 1 Survival of diploid *Artemisia tridentata* plantlets after 16 weeks of acclimation (sorted by genotype survival rate). This procedure was repeated twice for each ex vitro individual line using 11- and 16-week-old in vitro plantlets.

Individual line	Plantlets transferred	Age of in vitro plantlets (weeks)	Plantlets survived	Survival rate (%)
G2_b24_1	164	11	67	41
	86	16	30	35
G1_b2_1	171	11	18	11
	118	16	19	16

TABLE 2 Survival and average shoot water potential of diploid *Artemisia tridentata* plantlets after outplanting in the greenhouse (sorted by genotype survival rate). Values for shoot water potential represent the mean ± standard deviation based on surviving plantlets.

Individual line[a]	Plantlets transferred	Plantlets survived	Survival rate (%)	Average shoot water potential (Ψ_{shoot})
G2_b24_1	37	37	100	1.58 ± 0.12
G1_b2_1	37	11	30	0.89 ± 0.24

[a]Individual line G2 was 22 weeks of age and G1 was 32 weeks of age when data were collected.

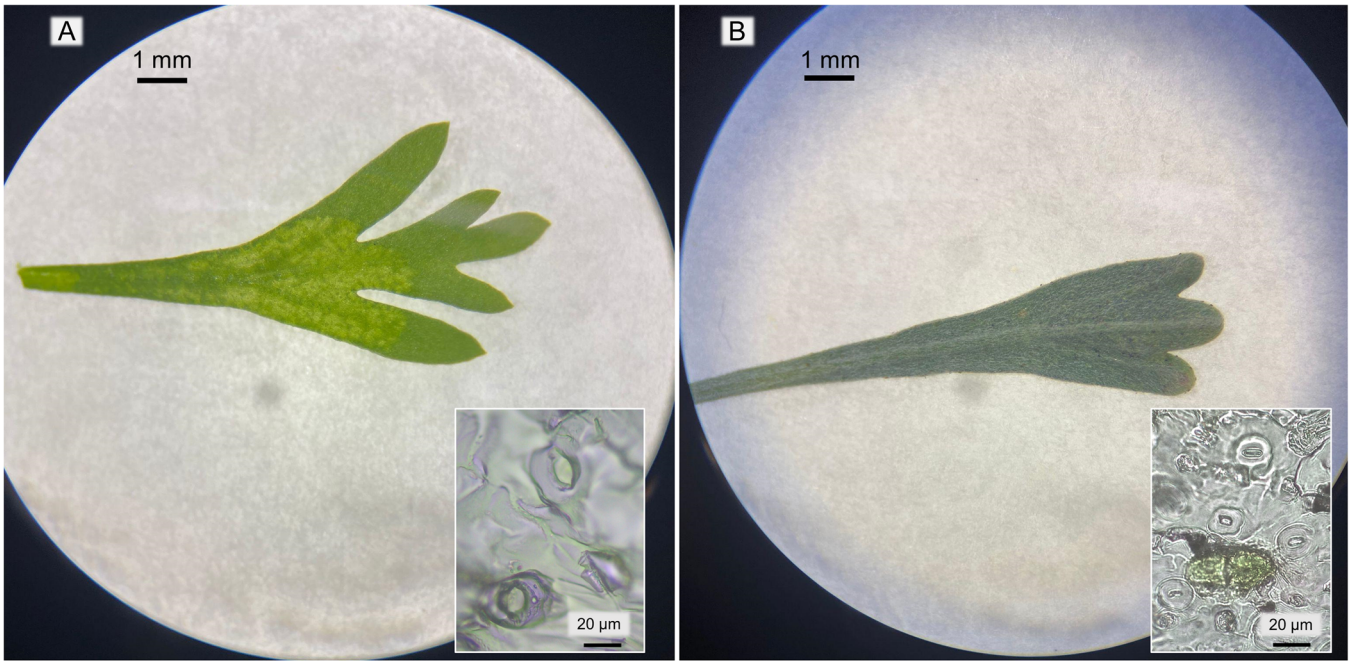

FIGURE 2 Morphological changes that occur during the acclimation and hardening process of diploid *Artemisia tridentata* leaves. (A) In vitro–grown leaf (thin cuticle, bright green color) and (B) ex vitro–grown leaf (trichomes, thicker cuticle layer). Leaves were imaged at 35× using a dissecting microscope. Insets on each panel represent stomata collected from each leaf type. Stomata for in vitro–grown leaves are larger and occur less frequently than on the ex vitro, adapted leaves (images collected at 40×).

abscission of in vitro leaves and development of new, adapted leaves (i.e., trichomes present, thick cuticle, smaller stomata) (four weeks); (iii) implementation of a watering regime that promotes the growth of functional plantlets (i.e., maintains water balance) (six weeks); and (iv) transfer of the plantlets into a more complex soil potting mix (composed of sand, silt, and vermiculite at 2:1.5:0.5 v/v/v; hereafter referred to as silty mix) in an open vessel (23% ± 5% humidity) to complete acclimation and especially root hardening (two weeks or longer depending on specific study needs). Upon completion of the 16-week protocol, the sagebrush plantlets exhibit a phenotype similar to sagebrush seedlings (Figures 1 and 2). The plantlets are now ready for the second step of the protocol.

Preparations for G×E experiments

To prepare plantlets for short-term (10–20 days) growth chamber G×E experiments, they were kept well-watered ($\Psi_{soil} > -0.1$ MPa) and acclimated to growth chamber conditions (16-h photoperiod, 24°C, and a light intensity of ~128 µmol m^{-2} s^{-1} using fluorescent lights) for one week in a Percival instrument (model CU-41L4C8; Percival Scientific, Perry, Iowa, USA). After this acclimation time, Ψ_{shoot} was measured (see methods below) to ensure that the plantlets can maintain, at least under well-watered conditions, Ψ_{shoot} compatible with CO_2 assimilation and growth (−2.5 MPa) (Kolb and Sperry, 1999). To prepare plantlets for mid- to long-term (6 months to 10 years) field experiments, they were transferred from large containers containing sandy soil into Deepot growing containers (2.7 × 14 in; Stuewe & Sons, Tangent, Oregon, USA) containing soil mix (1:1 v/v) composed of one part soil conditioners (1:2:1 volcanic cinder:vermiculite:peat moss) and one part greenhouse potting mix (1:1 top soil:compost) (Figure 1). After transplanting, plantlets were maintained in the greenhouse (16-h photoperiod, 23°C, 25% ± 5% relative humidity) and watered deeply every two days (i.e., water drips from the bottom of the pots) for about six months. Midday Ψ_{shoot} was measured from shoot tips covered with a reflective baggie and equilibrated for 20 min. Once equilibrated, the stem was excised near the petiole and measurements were collected using a Scholander-type pressure chamber (model 1505D; PMS Instrument Co., Corvallis, Oregon, USA).

Assessing survival rates

The 16-week acclimation and hardening of in vitro–grown sagebrush plantlets (taking place in a laboratory setting) resulted in low to medium survival rates reflecting genotypes (Table 1, Appendix S1). These results agree with previous findings, where a strong genotype effect was observed on rooting and growth rates of in vitro individual lines (Barron et al., 2020), and are comparable to other studies, especially in woody species exhibiting faster growth rates (e.g., 40–80% depending on treatments in American chestnut; Oakes et al., 2020). The outplanting of acclimated

and hardened plantlets in the greenhouse in preparation for field G×E experiments resulted in low (30%) to high (100%) survival rates, again reflecting genotypes (Table 2). Although the G1 individual line did not perform as well as the G2 individual line, we believe these results demonstrate the efficacy of this protocol to produce "seedling-like" sagebrush plantlets for G×E experiments. Although the sagebrush plantlets experienced somewhat high mortality (especially when compared with herbaceous species; Tables 1 and 2), this is not unusual when acclimating in vitro–grown plants to ex vitro environments (Shackel et al., 1990; Chandra et al., 2010).

CONCLUSIONS

Overall, our protocol provides a standardized method to acclimate and harden sagebrush plantlets from an in vitro environment to ex vitro conditions. This protocol yields an adequate number of plantlets for mid-scale G×E experiments (Tables 1 and 2) and produces plantlets that (i) are phenotypically similar to field-grown seedlings (Figures 1 and 2) and (ii) do not exhibit signs of water stress (Table 2). Finally, this protocol could be used as a benchmark for slow-growth, woody species occurring in dry ecosystems worldwide (Schenk et al., 2008; Drake et al., 2019) and contribute to ex situ conservation as well as fundamental research.

AUTHOR CONTRIBUTIONS

P.M., R.B., M.S., and S.B. conceived the research and designed the experiments; P.M. performed all the experiments. P.M. and S.B. analyzed the data and wrote the manuscript. All authors provided edits on the manuscript and approved the final version of the manuscript.

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DATA AVAILABILITY STATEMENT

The data for survival, shoot water potentials, volumetric water content, and soil matrix potential (sandy and silty soils) can be found in Appendix S1. The detailed, step-by-step protocol is available at protocols.io (dx.doi.org/10.17504/protocols.io.j8nlk4zpxg5r/v2; Martinez et al., 2022).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Appendix S1. Data for survival, shoot water potentials, volumetric water content, and soil matrix potential in this study.

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