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🌍 Acclimation of in vitro grown individual lines of big sagebrush (*Artemisia tridentata*) to ex vitro conditions in support of genotype-by-environment experiments and restoration V.3

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

<https://doi.org/10.1002/aps3.11515>

LINK

When you cite this protocol, please also cite the full paper.

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We use this protocol and it's working

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Disclaimer

This protocol has been tested and applied on *in vitro* diploid individual lines of *Artemisia tridentata* subsp. *tridentata* (using protocol described in Barron et al., 2020) and it might require minor adjustments to work on the other subspecies and cytotypes.

Citation

Barron, R; Martinez, P; Serpe, M.; Buerki, S
. Development of an In Vitro Method of Propagation for *Artemisia tridentata* subsp. *tridentata* to Support Genome Sequencing and Genotype-by-Environment Research.
Plants.

[10.3390/plants9121717](https://doi.org/10.3390/plants9121717)

LINK



Abstract

The following protocol describes the *ex vitro*, hardening and acclimation of *in vitro* grown genetically identical plantlets (i.e. clones) of *Artemisia tridentata* ssp. *tridentata* (Asteraceae). The goal of this protocol is to create functional clonal lines of *Artemisia tridentata* to be used in genotype-by-environment (GxE) experiments. Overall, the protocol consists of the following four major steps and takes 16 weeks to complete on 11-16-week old *in vitro* plantlets: i) transfer *in vitro* plantlets from modified Murashige & Skoog (MMS) media to a sand and vermiculite soil mixture (4:1 ratio; hereafter referred to as sandy soil) in an enclosed, high humidity vessel to initiate plantlet establishment and root growth (four weeks); ii) gradually open vessels to initiate dropping of *in vitro* leaves and growth of functional leaves (four weeks) associated with decreased of humidity and increased gas exchange; iii) establishing a watering regime to promote and maintain growth of functional plantlets (six weeks) and iv) transfer plantlets into a more complex soil mixture (similar to natural conditions composed of sand, silt and vermiculite at 2:1.5:0.5 ratio; referred to as silt soil) in an open vessel to complete acclimation; especially hardening of the root system (two weeks or more depending on specific needs). Upon completion, the sagebrush plantlets will be exhibiting a similar phenotype as sagebrush seedlings. Finally, although optional, we are encouraging users to conduct stem xylem pressure measurements on acclimatized and well-watered plantlets prior to starting GxE experiments to evaluate their hydraulic conductivity and overall level of stress.

When citing this protocol, please also cite the full paper.

Citation

Martinez P; Barron R; Serpe M; Buerki S. (2023)

. Acclimation and Hardening of a slow-growing woody species emblematic to western North America from *in vitro* grown plantlets.

Applications in Plant Sciences.

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LINK

Guidelines

- *In vitro* plantlets should be between 11 and 16 weeks old at time of transplant. Individuals younger than 11 weeks have insufficient biomass and root growth to withstand the acclimation. Individuals older than 16 weeks will have a more extensive root system and are at greater risk for root tearing during transplant procedure.



Materials

Equipment and Consumables

- Plant tissue culture chamber (Percival Scientific™ CU41L4)
- Medium/Large Steam Sterilizer (Autoclave)
- Laminar flow clean bench (Baker EdgeGARD HF)
- *PhytoCon* vessels (C221, 946ml) <https://phytotechlab.com/i-phyto-i-con-32-oz-946-ml.html>
- *PhytoCon* vessels (C215, 473ml) <https://phytotechlab.com/i-phyto-i-con-16-oz-473-ml.html>
- pH meter (VWR™ Cat. 101076-426)
- Colored tape (Fisher Scientific™ Cat. 15-901-10R)
- Autoclave indicator tape (Fisher Scientific™ Cat. 15-901-110)
- 2L Erlenmeyer flask
- 500 mL graduated cylinder
- Stir plate w/ stir bar
- Dropper (Fisher Scientific™ Cat. 14-955-500)
- Balance (Ohaus SP-6000 Scout Pro balance, 6000g x 1g)
- Balance (Ohaus Scout STX Portable Balances Fisher Scientific™ Cat. 01-922-411)
- Weigh boat or weigh paper (Fisher Scientific™ Cat. 18-001-26)
- Spatula (Fisher Scientific™ Cat. 14-357Q)
- 1 mL pipette w/tips
- Play sand (Home Improvement Stores)
- Vermiculite (Gardening Stores)
- Silt (VWR™ Cat. 470025-202)
- Greenhouse soil mix (top soil/compost mix) (Gardening stores)
- Soil conditioners (Volcanic cinder) (Gardening stores)
- 983cm³ Deepot containers (Greenhouse megastore)
- Plastic tote (20" x 15" x 5" or similar)
- Forceps (Fisher Scientific™ Cat. 50-118-3908)
- Scoop (Fisher Scientific™)
- Large PYREX™ reusable petri dishes (Fisher Scientific™ Cat. 08-747F)
- Bead Sterilizer (VWR™ Cat. 75999-328)
- EC-5 Soil Moisture Sensor (METER Group, 40593-S)
- Teros 21 Gen 2 Water Potential Sensor (METER Group, 40271-S)

Optional equipment

- Scholander type Pressure chamber (PMS Instruments 1505D).

Solutions and Reagents



- Murashige and Skoog w/ Gamborg vitamins: M404; Phytotechnology laboratories; phytolab.com; powder <https://phytotechlab.com/murashige-skoog-modified-basal-medium-with-gamborg-vitamins.html>
- Preservative for Plant tissue culture Media: plant cell technology; plantcelltechnology.com; liquid <https://www.plantcelltechnology.com/plant-preservative-mixture-ppm/>
- Potassium Hydroxide 0.1M: Fisher Scientific Cat. AC611150010; liquid <https://www.fishersci.com/us/en/browse/80013557/potassium-hydroxide.html>
- Ethanol (190 proof), USP grade, (Boise State University Chemistry Department)
- Hoagland Modified Basal Salt Mixture: H353; Phytotechnology laboratories; phytolab.com; powder <https://phytotechlab.com/hoagland-modified-basal-salt-mixture.html>

Troubleshooting

Prepare sandy soil with nutrient solution

4h 25m

1 Prepare sandy soil

30m

1. Mix play sand and vermiculite to a 4:1 v/v (sand/vermiculite) in large plastic tub (hereafter referred to as sandy soil). If play sand is very dry, add enough DI water to moisten the sandy soil mixture.

Note

Sand/vermiculite soil mixture will slightly hold form when pressed into a ball.

2. Tare *PhytoCon* vessel (946mL) on the balance and add 406 g of the sandy soil mixture.

Note

The soil mixture should reach a height of ca. 6 cm (Fig. 1).

2 Media preparation

30m

1. Using a graduated cylinder, add 500 mL DI water to an Erlenmeyer flask with stir bar and place onto the stir plate. Turn on stir plate to ~300 rpm, no heat is needed.
2. On the balance, weigh out 2.2 g Murashige and Skoog + Gamborg vitamins into weigh boat and add to flask. Mix until dissolved.
3. Pipette 1 mL Plant Preservative Mixture (PPM) to flask. Add 500 mL DI water to media mixture to bring to 1L volume.

3 pH correction of the media

10m

1. Place calibrated pH meter into the 1/2MS + PPM solution created in Step 2.
2. Add 0.1M KOH dropwise to the solution until pH 5.8 is reached.

Note

The solution has little buffering capacity so wait for pH meter to stabilize before adding more 0.1M KOH. 5 drops at a time works well at this volume.

4 Add media to sand mixture and autoclave



20m

1. Pour 40 mL of the 1/2MS + PPM solution into a graduated cylinder and pour into prepared *PhytoCon* vessels.
2. Place lids lightly on top of vessels.

Note

Do not firmly place lids until after steam sterilization procedure due to expansion of moistened soil.

- 4.1
- Steam sterilize the prepared *PhytoCon* vessels at 15 psi, 121°C, for one hour to sterilize the soil and vessels.
 - After sterilization process, press lids down tightly.
 - Allow soil + media to cool thoroughly before beginning transplant procedure (step 5 onwards).

2h

Transfer plantlets from *in vitro* to *ex vitro* conditions

3h 20m

5 Sterilize and prepare Laminar flow clean bench for transplant of *in vitro* plantlets (Fig. 1)

20m

1. Sterilize the inside of the laminar flow clean bench with 70% v/v ethanol. Wipe the outside of the culture vessels containing *in vitro* grown plantlets and *PhytoCon* vessels with 70% v/v ethanol prior to placing inside the laminar flow clean bench. Pour a small amount of 90% v/v ethanol into large petri dishes and allow to dry in the laminar flow clean bench. Place forceps and scoop into 90% v/v ethanol. Heat bead sterilizer to 280°C.



■ Fig 1. *PhytoCon* vessels and *in vitro* grown plantlets prior to transplant.

6 Transplanting *in vitro* grown plantlets into *ex vitro* conditions (Fig. 2)

3h



1. Working inside the laminar flow clean bench, sterilize scoop by dipping it into 90% v/v ethanol and placing in bead sterilizer for ~30 seconds. Open *PhytoCon* vessel and dig a small ~3 cm deep hole into the soil medium.
2. Rinse scoop with DI water to remove excess soil and place back into 90% v/v ethanol. Then, sterilize forceps using the same procedure used to sterilize the scoop.
3. Allow forceps to cool slightly.
4. Open the culture vessel containing an *in vitro* grown plantlet and grasp the base of the stem (right above the sterile media). Gently pull the plantlet and roots out of the media (Fig. 2).
5. If media clings to the roots, place into sterile petri dish and using forceps, scrape off media.

Note

CAUTION: Take care not to rip roots at this step.

6. In the *PhytoCon* vessel, feed roots into the small whole previously dug into the soil media. Gently cover roots, and press soil down lightly. Replace lid tightly and label vessel.
7. Continue steps until all plantlets are transplanted.

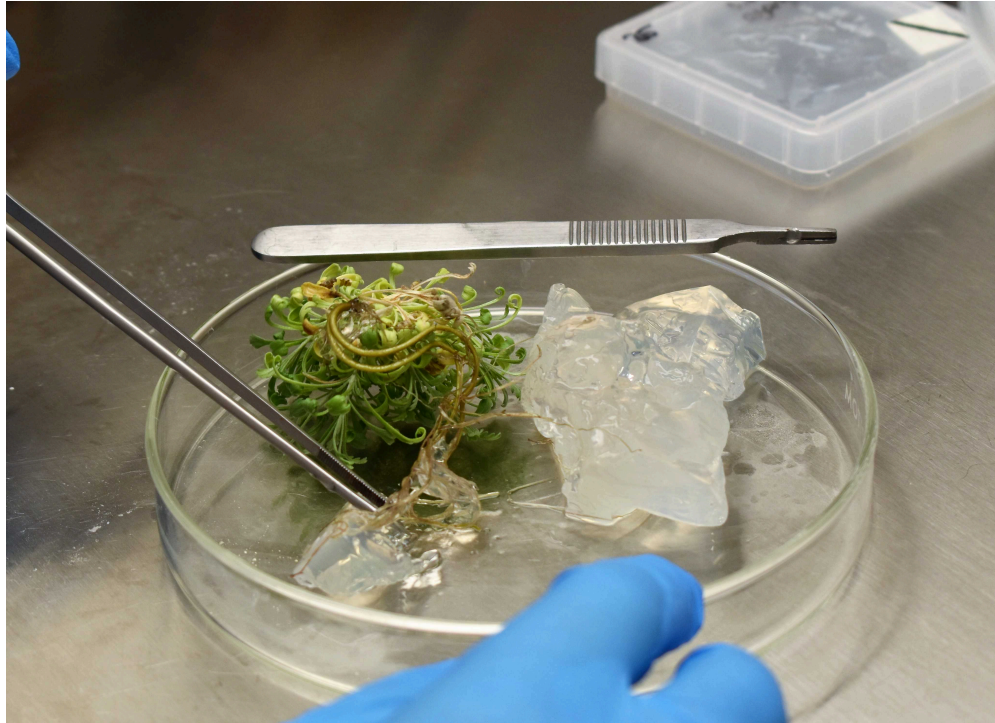


Fig 2. Removing media from in vitro grown plantlet.

Acclimation Phase

14w

7 Plant Tissue Culture Chamber (Fig. 3)

2w

- After plantlets are transplanted, place *PhytoCon* vessels into the plant tissue culture chamber to begin acclimatization. Tissue chamber environment should be at 24°C and PAR $\sim 128 \mu\text{mol photons} \times \text{s}^{-1} \times \text{m}^{-2}$ (= same abiotic conditions as with the in vitro plantlets). The particular chamber pictured uses fluorescent bulbs.

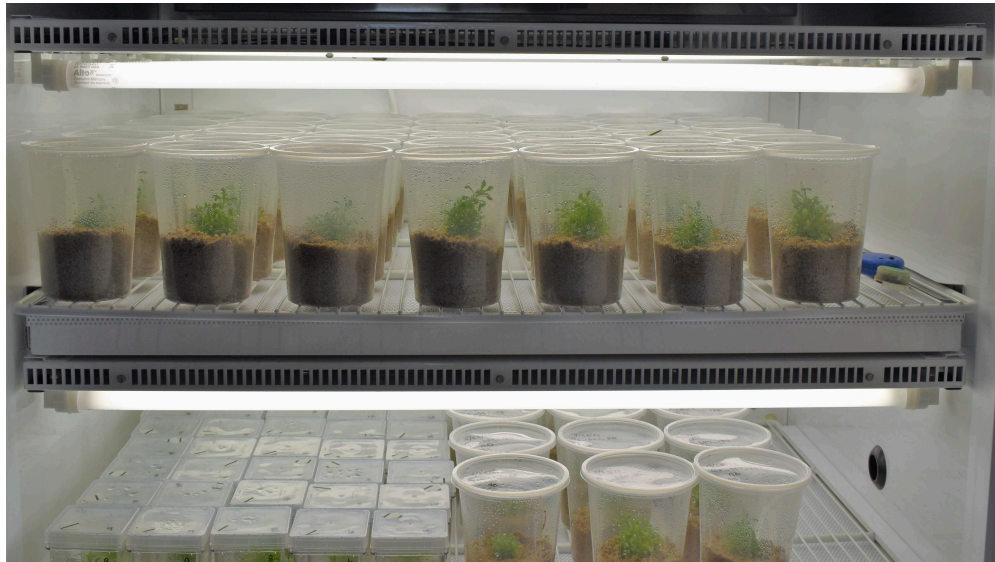
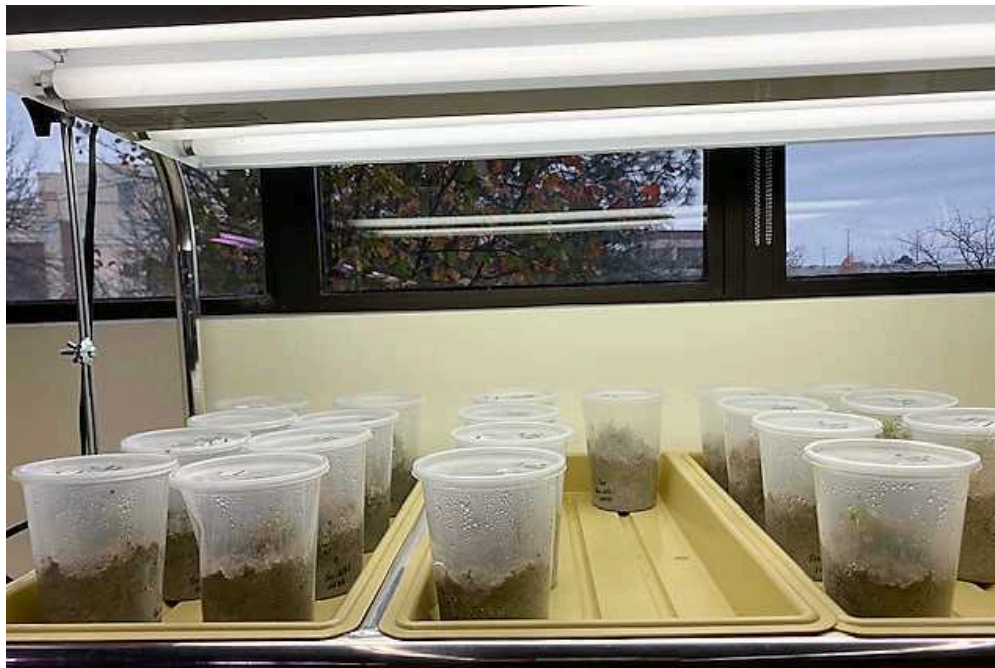


Fig 3. Explanted plantlets inside the plant tissue culture chamber.

7.1 Move closed vessels into the lab (Fig. 4)

2w

- Move closed *PhytoCon* vessels into the laboratory to adjust plantlets to variable light intensity. Growth lights are fluorescent bulbs with PAR $\sim 82 \mu\text{mol photons} \times \text{s}^{-1} \times \text{m}^{-2}$ and temperature is between 22 and 24°C. Vessels stay closed during this 2-week period and you will observe condensation on the sides (Fig. 4).



- Fig 4. Closed *PhytoCon* vessels with explanted plantlets; adjusting to variable light intensity. Plantlets are maintained at 99% humidity.

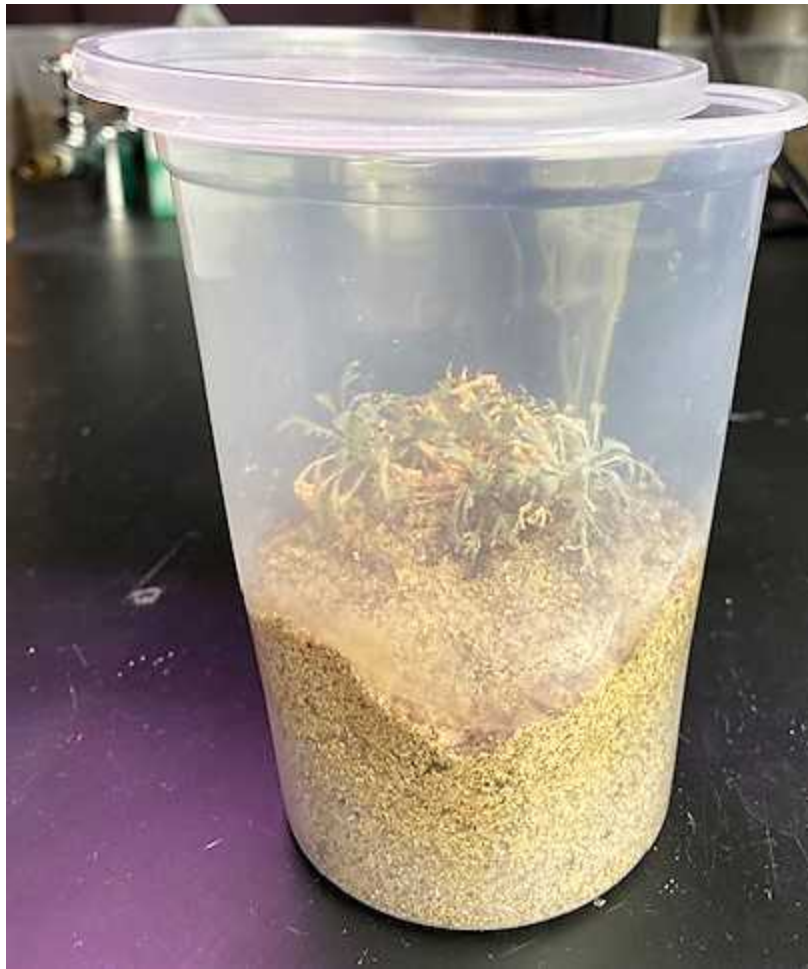
7.2 Crack open vessels in the laboratory (Fig. 5)

4w

- Open *PhytoCon* vessels (see Fig. 5) to allow for gas exchange to occur. Remove lids and replace slightly off center to minimize water loss and encourage phenotype changes (morphogenesis) to occur.

Note

In vitro leaves will drop and newly adapted leaves will set (Fig. 5).



- Fig 5. Explanted plantlet with *PhytoCon* lid opened and placed off-center to allow for gas exchange and phenotype change.

7.3 Fully open vessels and water as needed (Figs. 6,7, 8)

6w



- During this step, lids will be fully removed as determined by plantlet morphology change (see Fig. 6,7 for an example), if there is not a sufficient phenotypic change (= new adapted leaves, which are sage color and covered of trichomes), removing the lid completely will cause plantlet death.

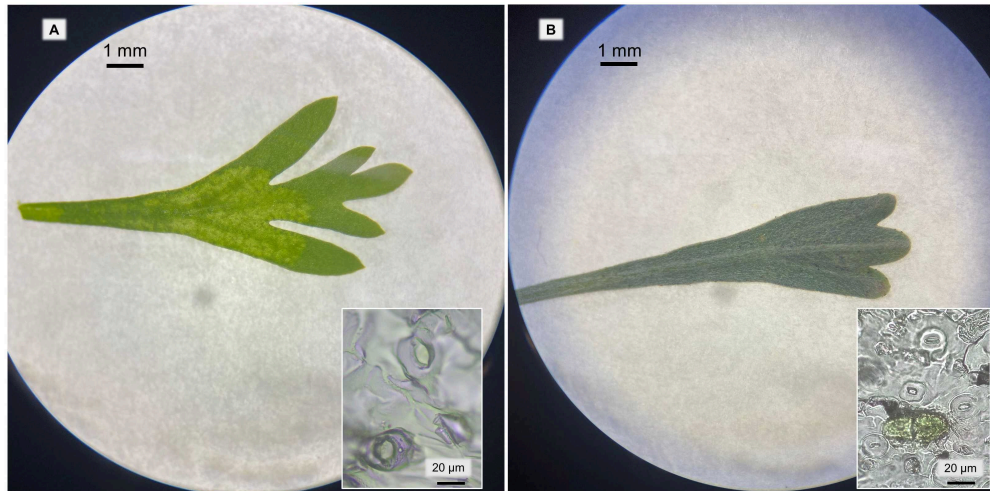


Fig 6. Image demonstrating morphological changes that occur during acclimation and hardening process. Panel A; *in vitro* grown leaf (thin cuticle, bright green color) and B; *ex vitro* leaf (trichomes, thicker cuticle layer). Leaves were imaged at 35x using dissecting scope. Insets on each panel represent stomata collected from each leaf type. Stomata for *in vitro* leaves are larger and occur less frequently than on the *ex vitro*, adapted leaves (images collected at 40x).

Note

~60% of plantlet leaves must exhibit change in color and appearance before completely removing lid.

- Water will be given on an as needed basis when soil water potential falls below -0.2 MPa and no more condensation can be seen on the vessel walls.
- Plantlet can be maintained at this stage indefinitely, watering ~50mL 2-3 times per week. We advise using Teros-21 water potential probes to maintain soil water potential under -0.5 MPa (Fig. 8).

Note

Be careful here to maintain a strict watering regime since high evapotranspiration will rapidly cause soil water depletion, resulting into plantlet death by cavitation. Also, fully saturating soil could lead to an anoxic environment for the roots as well as cause fungal infections or outbreaks of other pathogens.

- After hardening (ca. 15 weeks of growth), water with 1/2 strength Hoagland modified basal salt mixture one time per week.

Note

Fungal growth on dead and dying maladapted leaves may become a problem at this stage (once watering regime begins). To minimize this, water away from the plantlet, along the sides of the soil and vessel.



Fig 7. Plantlet undergoing phenotype change (morphogenesis) during acclimatization phase of ex vitro procedure. Notice trichomes (giving sage color) on the new leaves located on the crown of the plantlet.

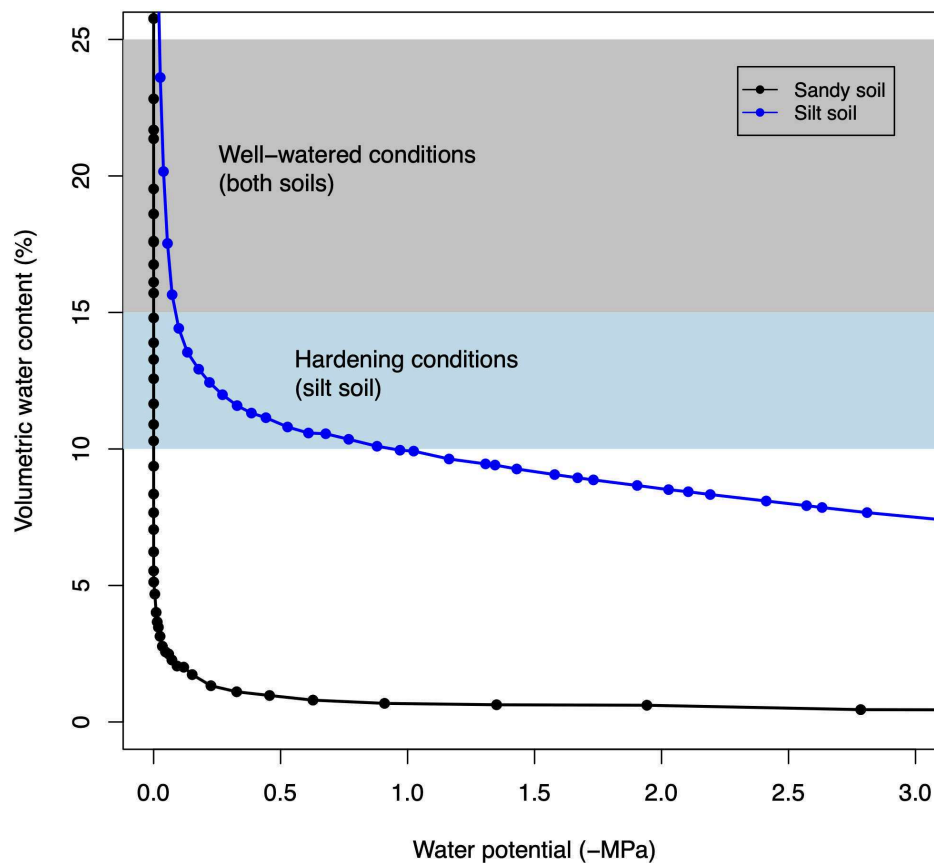


Fig 8. Soil water retention curves for the sandy and silt soils in closed containers (inferred using EC-5 and Teros 21 METER probes). Hardening conditions occur between -0.2 and -0.9 MPa and well-watered is above -0.1 MPa.

Completing the hardening process

2w 0d 2h 20m

8 Prepare silt soil

20m

1. Mix sand/silt/vermiculite (2:1.5:0.5 v/v/v) in large a plastic tub (referred to as silt soil). Add enough DI water to just moisten soil.
2. Fill smaller *PhytoCon* vessel (473 mL) approximately 3/4 of the way full with the silt soil mixture (Fig. 8).

**Note**

- The smaller *PhytoCon* vessels promote acclimatization of plantlets by alleviating any microclimate occurring in the headspace of the larger *PhytoCon* vessels and also supports physiological measurements (Fig. 8).

8.1 Transfer plantlet into silt soil

2h

1. Gently remove plantlet from larger *PhytoCon* vessel (946 mL). Roots are fine at this stage and tear easily.
2. Place plantlet in smaller *PhytoCon* vessel (473 mL) containing the silt soil mixture. Once plantlet is in, fill to the top with additional soil.
3. Water with 1/2 strength Hoagland Modified basal salt mixture until soil is moist, but no standing water is observed.

8.2 Hardening root system (Fig. 8)

1w

- The soil water potential should be kept between -0.2 MPa and -0.9 MPa (using Teros-21 METER probe) to harden roots and complete acclimatization (Fig. 8).

Note

Falling under -2.0 MPa soil water potential will create embolism and plantlets might die from cavitation (Fig. 8).



Fig 8. Fully hardened and acclimated plantlet. Note the cable on the side showing where the EC-5 METER probe is inserted to monitor soil water content (Fig. 8).

Note

After this step, plantlets are acclimatized. We suggest following the instructions in Step 9 to prepare plantlets for controlled laboratory GxE experiments or transplant plantlets into open bottom containers for longer term maintenance in a greenhouse environment to be used in field experiments.

Prepare plantlets for controlled laboratory GxE experiment

1w 0d 1h

9 Well-water plantlets

1w

- Maintain the soil water potential below -0.1 MPa using Teros-21 METER probes to ensure plantlets are not stressed (Fig. 8).

9.1 Acclimate plantlets to growth chamber conditions

1w

- Set plant tissue growth chamber conditions to a 16-hr photoperiod, 24°C and PAR $\sim 128 \mu\text{mol photons} \times \text{s}^{-1} \times \text{m}^{-2}$ under fluorescent bulbs.
- Continue with watering regime (using Teros-21 probes) to keep plantlets in well-watered conditions (see step above and Fig. 8).

9.2 Measure shoot water potentials

1h

Measuring shoot water potentials verifies plantlets are not experiencing water stress.

1. Follow instructions on the pressure chamber to properly attach gas hoses to the Scholander-type pressure chamber.
2. Measure plantlet during mid-day.
3. Cover leaves with reflective material and allow to equilibrate for 20 minutes.
4. Leaving leaves covered, excise a stem from the plantlet using a razor blade.
5. Feed stem through the gasket from the pressure machine. Once stem is fed through, tighten down the gasket and place back onto the pressure chamber.
6. Turn the lever to chamber and adjust the rate valve to a rate that makes it easy to record values.
7. When the end of the stem is wet, record the pressure displayed on the screen.

Note

Well-watered plantlets should have shoot water potentials around -1.2MPa. Note that such value would be alarming for other plant species, but it is normal/acceptable for sagebrush.

Prepare plantlets for field GxE experiments

3h

10 **Transfer plantlets to new vessels and soil mix**

1. Gently remove plantlets from large *PhytoCon* vessels and transfer to a (1:1 v/v/v) soil mix comprised of one part soil conditioners (one-part volcanic cinder: two-parts vermiculite: one-part peat moss) and one-part greenhouse potting mix (one-part top soil: one-part compost) into 983cm³ Deepot containers (Fig. 9).
2. Move plantlets into greenhouse with 16-hr photoperiod maintained at 23° C.
3. Water plantlets deeply (i.e. water drips from the bottom of the pots) every two days.

Note

Deepot containers have open bottoms (unlike *PhytoCon* containers) therefore, water can drain freely and this reduces the change of overwatering and creating anoxic conditions for the root system.



Figure 9: Six month old plantlets in Deepot containers in greenhouse conditions (16-hr photoperiod, 23° C). Soil mix is 1:1 greenhouse potting soil and soil conditioners. Plantlets are watered deeply (i.e. water drips from bottom of the pot) every two days.

Citations

Martinez P; Barron R; Serpe M; Buerki S.. Acclimation and Hardening of a slow-growing woody species emblematic to western North America from in vitro grown plantlets

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