

AN IMPROVED PROTOCOL FOR

# Micropropagation of Saltbush

(*ATRIPLEX*) SPECIES

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Photo by Ruth Sedillo

## ABSTRACT

*Atriplex* species (Chenopodiaceae) include a diverse genera of saltbushes found worldwide and valued for forage, arid land restoration, remediation, and halotolerance. Within the genus, the species *A. canescens* (Pursh) Nutt. (fourwing saltbush) is distributed throughout arid western regions of North America where it is a popular restoration and forage species. *Atriplex torreyi* (S. Watson) S. Watson var. *griffithsii* (Standl.) G.D. Br. (Griffiths' saltbush) is a threatened subspecies found only in southern regions of New Mexico and Arizona. Micropropagation of either species may result in formation of *hyperhydricity*, or vitrification, which is an induced physiological disorder and a common problem of *in vitro* cultured plants. Leaves and stems of hyperhydric plants are thick, distorted, and brittle with a glassy (vitrified) appearance. This physiological disorder may be induced by culture conditions that provide excess inorganic nitrogen or high humidity. The protocol described herein uses commercially available, low nitrogen nutrient medium and uses vented lids to reduce humidity within culture vessels. Shoot multiplication can be initiated from either apical shoots or seeds. This protocol has been successfully applied to micropropagation of both species, offering an improved alternative for *Atriplex* propagation.

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### KEY WORDS

*Atriplex canescens*, fourwing saltbush, *Atriplex torreyi*, Griffiths' saltbush, hyperhydricity

### NOMENCLATURE

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**A**triplex comprise a genus of more than 400 species within the family Chenopodiaceae. These species are distributed worldwide across arid and semiarid zones (Sanderson and McArthur 2004; Ortiz-Dorda and others 2005). Most *Atriplex* are classified as “saltbushes” due to their salt tolerance. This makes *Atriplex* species popular candidates for plant reclamation projects in saline and arid areas (Glenn and Brown 1998; Glenn and others 1998). *Atriplex* species are also highly productive range forages in the arid and semiarid southwestern US (Cibils and Swift 2003; Sanderson and McArthur 2004) and are known to contain a variety of seedborne endophytes that likely contribute to host hardiness in arid lands (Barrow and others 1997; Lucero and others 2006).

For many years, we have successfully micropropagated fourwing saltbush using a Murashigie and Skoogs (MS)-based medium that is modified with complex organic nitrogen sources (Reyes-Vera and others 2008). As prepackaged medium formulations have become increasingly available, we were interested in finding a medium that could be used without alteration of the nitrogen source. Subjective evaluation of existing fourwing saltbush lines suggested shoot cultures could be successfully maintained on Woody Plants Medium (WPM) (Lloyd and McCown 1980). When Griffiths’ saltbush (*Atriplex torreyi* (S. Watson) S. Watson var. *griffithsii* (Standl.) G.D. Br.), a threatened species, was added to our *in vitro* research collection, we used the opportunity to optimize growth regulator treatments and develop this *Atriplex* micropropagation protocol based on WPM. Best results for this and any micropropagation project will be obtained under aseptic conditions in a clean laboratory with a biosafety cabinet or laminar flow hood to minimize sample contamination. In addition, an autoclave is needed to ensure sterility of all media and utensils that come in contact with the plant material.

## STARTING MATERIAL

*Atriplex* species tend to be prolific seed producers. Seeds of fourwing saltbush ripen in late fall and are often retained on the plant throughout the winter and into the spring. Hence, the window of opportunity for seed collection is large. If seeds are unavailable, however, apical shoots can be substituted. In other reports, leaves, auxiliary buds, and even roots have provided starting material for *Atriplex* micropropagation, though doing so may require modification of the growth regulators added to the basal medium (Mei and others 1997; Reyes-Vera and others 2008).

## DISINFESTATION

Seeds should first be excised from the surrounding bracteoles and utricles to eliminate these sources of contaminating microbes. This can be readily accomplished by hand using a sterile pair of nail clippers. The excised seeds can then be surface disinfested by shaking them in a 1:100 solution of Zeroto™ (Biosafe Systems LLC, East Hartford, Connecticut) and sterile water for 10 min.

If using apical shoots or other tissues, rinse them under running tap water, brushing gently with your fingers to remove any visible dirt or particles, then disinfest in Zeroto™ as described above.

Traditional disinfestation protocols using sequential exposure to ethanol and hypochlorite or hydrogen peroxide solutions are also effective. We have successfully disinfested *Atriplex* tissues by immersing in 95% ethanol for 1 min and then in 2.6% sodium hypochlorite for 7 min prior to rinsing 3 times in sterile distilled water. Recently, we have found the Zeroto™-based disinfestation to be more effective for removing heavy microbial infestations and to be less damaging to plant tissues. Since the phytotoxicity of Zeroto™ is low, and

the diluted product degrades rapidly, we can transfer tissues directly from the disinfectant solution to the tissue culture media without the need for rinsing. A notable disadvantage to using Zeroto™, which is packaged and marketed for industrial-scale use, is that it is only packaged in large quantities. Hence, if it is being used a few milliliters at a time to prevent microbial contamination of tissues prior to micropropagation, it can be expensive to purchase and store.

## SHOOT MULTIPLICATION

Disinfested seeds or apical shoots are transferred aseptically to shoot multiplication medium consisting of 2.4 g/l Woody Plant Media with vitamins (PhytoTechnology Laboratories®, Shawnee Mission, Kansas 66282-2205, product L449), 30 g/l sucrose, 5 mg/l 6-( $\gamma/\gamma$  dimethylallylamino) purine (2iP), and 0.8% plant tissue culture grade agar. The pH should be adjusted to  $5.6 \pm 0.05$ . Polycarbonate culture boxes with vented lids containing a 10 mm (0.4 in) central opening covered with a polypropylene membrane (0.22  $\mu$ m pore size) are utilized to reduce the buildup of excess humidity in the culture vessels. High humidity is thought to contribute to the formation of hyperhydrated tissues (Reyes-Vera and others 2008). Culture boxes can be incubated at  $28 \pm 1$  °C (82 °F) under fluorescent light on a 16-h day, 8-h night cycle with a light intensity of 14 to 18  $\mu$ mol/(m<sup>2</sup> · s). After 30 d, multiple clonal shoots and some basal callus should be present. Shoots can be aseptically excised and subcultured to fresh medium to generate additional shoots. If callus maintenance is desired, callus can be transferred to medium containing WPM basal salts, 30 g/l sucrose, 0.75 mg/l picloram, and 2 mg/l 6-benzylaminopurine (BAP).

## ROOT INDUCTION

Shoots excised from multiple shooting clusters can be transferred to hormone-free WPM medium (prepared as for shoot multiplication medium but omitting 2iP) and cultured under the same light and temperature conditions as described above. Plants should be transferred to fresh medium every 30 d. Root initials should appear within 30 d and rooted plants should be ready for acclimatization in 60 d.

## ACCLIMATIZATION

Rooted plantlets should be gradually acclimatized to a standard greenhouse environment. This can be accomplished with minimal root disturbance by autoclaving hydrated peat pellets (Jiffy Products of America Inc, Lorain, Ohio) inside the vented culture boxes and aseptically transferring rooted shoots to the peat pellets. Maintain plantlets in growth chambers until new root growth is clearly evident along the edges of the peat pellets (Figure 1C). At this time, the peat pellets can be transferred to standard potting soil, irrigated with tap water, and transferred to a greenhouse. Our greenhouse, located at Las Cruces, New Mexico, at lat 32.34°N, long 106.76°W, falls within USDA hardiness zone 8 (Cathey 1990). Temperature is maintained at 25 +/- 5 °C (77 °F) with natural daylight filtered through glass panels that are whitewashed in the summer. Average summer light intensity measured in the greenhouse is 820  $\mu\text{mole}/(\text{m}^2 \cdot \text{s})$ .

For potting soil, we typically use a medium rich in organic matter, such as Metro Mix® 360 (Sun Gro Horticulture, Canada Ltd, Vancouver, British Columbia) and do not add fertilizer. We have maintained rooted plants in the greenhouse for up to 12 mo without observing any obvious signs of nutrient deficiencies. Saltbushes, which are adapted to nutrient poor soils, appear to secure adequate nutrients from the growth medium without a need for supplemental fertilizer.

Plants undergoing acclimatization should be monitored closely; they will be ultrasensitive to minor changes in the environment until they have hardened. Greenhouse-acclimatized plants have been successfully established in outdoor settings. Six regenerated Griffiths' saltbush plants were recently donated to the Rio Grande Botanic Garden in Albuquerque, New Mexico, where they are to be included in an outdoor desert plants exhibit.

## SUMMARY

Halotolerant saltbushes are popular species for arid land restoration. We have simplified methods for micropropagating saltbushes by utilizing a low-nitrogen nutrient medium and vented lids to prevent hyperhydricity (Debergh and others

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Figure 1. Griffiths' saltbush. Multiple shoots produced by a single explant following treatment with 1.14  $\mu\text{M}$  IAA and 24.61  $\mu\text{M}$  2iP (A). Roots developing on the end of a micropropagated shoot and penetrating agar-based medium (B). Rooted explant in peat pellet (C). Mature, greenhouse-acclimated clonal plant (D).

Photos A–C by Isaac Reyes-Vera; D by Ruth Sedillo

1992). With this method, we have successfully initiated shoot multiplication lines from both seeds and apical shoots. Shoot cultures can easily be rooted and acclimatized to greenhouse conditions. Acclimatized plants have been successfully outplanted. Micropropagation provides a viable alternative to seed-based methods for producing *Atriplex*. These techniques may be particularly useful for protecting threatened species such as Griffiths' saltbush, for which viable seed supplies may be limited.

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