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An axenic plant culture system for Sporobolus alterniflorus



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We use this protocol and it's working

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Abstract

Sporobulus alterniflorus is a native grass that dominates intertidal salt marsh platforms along thousands of miles of the U.S. East and Gulf coasts. This grass exhibits tolerance to a broad range of abiotic stresses, including high salinity, anoxia, and high concentrations of toxic sulfide.
This species plays a critical role as a keystone plant in many salt marsh ecosystems. Its three-dimensional physical structure serves as a resource that fuels food webs both above and below ground, while its presence also modifies the environment for other components of the ecosystem.
The *in vitro* culturing of *S. alterniflorus* is often hindered by the difficulty of removing bacterial and fungal contaminants from its surfaces and tissues. Rhizomes, although easy to culture, are hollow structures heavily colonized by bacteria. Similarly, seeds are frequently colonized by fungi. An elegant alternative approach previously described in the literature involves generating somatic embryos from immature inflorescences. However, this protocol is complex and time-consuming.
In this study, we present a protocol for generating axenic *S. alterniflorus* plants derived from seeds. Our method offers several advantages, including high efficiency and a notable simplification compared to the current protocols used to establish vitroplants from somatic embryos.

Image Attribution

E.L.P.

Guidelines

This protocol includes several steps, including the collection of seeds in the field, seed germination, and in vitro establishment of cultures. Multiple media can be used to grow *Sporobulus* plants, here we selected a modified MS media that had a positive effect both in root adventitious regeneration and lateral shoot development without altering the morphology of the plant.

Materials

Materials

- 1 L plastic containers.
- Aluminum foil.
- Tweezers.
- Surgical blades.
- 50 mL conical tubes.
- Petri dishes.
- Pyrex 1L bottles.
- ~1 L glass culture vessels.

Reagents

- Soil.
- Bleach.
- Autoclaved water.
- Modified Murashige and Skoog medium.
- Indole-3-acetic acid (IAA).
- Kinetin.
- Agar.
- Sucrose.

Equipment

- Growing lights.
- ph Meter.
- Autoclave.
- Tissue culture hood .

Safety warnings

This protocol uses bleach. Hazard Statements. Bleach causes severe skin burns and eye damage and causes serious eye damage

Ethics statement

No animals or humans are used in this protocol.

Before start

Before completing any of the steps, you consult with your institution about any procedures related to soil processing and disposal.

Seed storage and germination

1 The plant material in this protocol is seedlings germinated ex vitro. While optional, we recommend autoclaving the soil and completing surface sterilization of the seeds to minimize the contaminants in the samples. Alternatively, seeds can be germinated in open containers.

1.1 **Seed Storage** 30m

- In the field, separate mature seeds from flower stalks.
- Transfer to a clean zip lock bag.
- Add water to maintain the seeds always wet.
- Store at 4°C, in the dark.
- Properly stored seeds should be viable for 6-12 months.

1.2 **Preparation of containers for seed germination** 3d

- Fill autoclavable containers to 1/4 of their volume with soil.
- Add water until the soil is completely saturated.
- Cover the containers' openings with aluminum foil.
- Autoclave for 1 h.
- After 48h, repeat the autoclaving step.
- Let containers cool down to room temperature before sowing the seeds.

1.3 **Seed preparation and germination (surface sterilization)** 2w

- Place seeds (with the glumes, see Fig.1) in a conical tube (50 mL)
- Add 40 mL of a dilution of commercial bleach to 20%.
- Transfer tubes to a tissue culture hood.
- After 20 minutes, remove the bleach solution.
- Add 40 mL of autoclaved distilled water and incubate 5 minutes.
- Remove the water, and repeat the rinse step two more times.
- Still in the hood, open the autoclaved soil containers and place seeds in the soil.
- Cover.
- Place containers in an illuminated culture chamber with a 16h light: 8h dark regimen, light intensity of ~ 100 µE, and a temperature of 20°C.
- Seedlings will germinate 1-2 weeks later.

DO NOT ALLOW THE SOIL TO DRY. Add autoclaved water as needed.

Note

We opted to include a high number of seeds in each container. It is important to note that the germination rate of Sporobolus alterniflorus seeds is frequently low, and our objective in this study was not to optimize the germination rate. We observed a sequential germination pattern among the seeds, so having a high number of seeds per container ensured a continuous supply of seedlings.
Additionally, we decided *to utilize seeds with intact glumes as our starting material. It is worth mentioning that manually removing the glumes did not have a significant impact and considerably increased the workload.*



Fig.1- Mature seeds (spikelets) before surface sterilization. On the right, seeds (caryopsis) after removal of external covers (glumes).

Removal of root system

2 Seven to 10 days after germination, seedlings should have well-developed aerial and root sections. The aerial part should be around 5 cm, robust-looking, and deep green. The radicle should emerge from the opposite end of the seed. Additional roots, developed from the crown area, might or not be present.



Fig.2- Smooth cordgrass seedlings.

2.1 **Remove seedlings from the soil.** 10m

- Gently pull seedlings from the soil.
- Place in autoclaved, room temperature water.

- Remove dirt.
- Place seedlings in a clean working area.

https://www.youtube.com/embed/WH3_Qu8ydww?controls=0

2.2 **Remove the radicle from the seedling**

10m

The primary root and endosperm of the seedlings are carriers of endogenous fungal contaminants.

- Use tweezers to remove the external covers of the seed, seed endosperm, and primary root.
- Use a clean blade to remove any root tissue under the crown area.
- Maintain, if present, the secondary roots formed in the crown area.

https://www.youtube.com/embed/pAjMeHl4AyU



Fig.3- Removal of root tissues.

Surface sterilization of trimmed seedlings

3 **Reagents**

50 mL conical tubes.

20% solution of commercial bleach.
Autoclaved distilled water.

3.1 Surface sterilization

25m

- Place 10-15 trimmed seedlings in a 50 mL conical tube.
- Add 40 mL of a 10% solution of commercial bleach.
- Cap the tubes.
- Mix gently.
- Incubate at room temperature for 20 minutes.

https://www.youtube.com/embed/b8LWLeOTnTU

Note

A bleach solution of 10% is usually strong enough to remove any possible bacterial and fungal contaminants in previously surface sterilized seeds grown in autoclaved soil. For seedlings growing in untreated soils, **we recommend using a 20% solution** of commercial bleach.

3.2 Rinsing

20m

- In the hood, pour out the bleach solution.
- Remove any excess bleach solution by rinsing the tube with autoclaved distilled water.
- Add 30-40 mL of autoclaved, room-temperature distilled water.
- Gently mix.
- Incubate for 5 minutes.
- Rinse the trimmed seedlings 2 more times in autoclaved, room-temperature distilled water.

https://www.youtube.com/embed/bC5T9st1RTc

Inducing new roots in surface sterilized trimmed seedlings

4 Root induction

We have removed the radicle of the seedlings to remove the endogenous fungal contaminant. To successfully grow the seedling into mature plantlets we need to induce a new root system. We will induce direct organogenesis in the crown area of the seedling using a combination of hormones.

4.1 Root induction medium (prepare in advance)

2h

Plant culture medium (MS)

This culture medium is a modification of the Murashige and Skoog (1962) and can be bought as dry powder from Phytotech Labs (**reference M527**).

- Add 4.43 g of powder to a 1L autoclave bottle.
- Add 30 g/L of sucrose for a final concentration of 3%.
- Add double distilled water for a final volume of 1L.
- Adjust the pH to 5.8.
- Add 8 g/L of agar.
- Autoclave for 40 minutes.

In the hood, add ~25 mL into as many 90 mm sterile Petri dishes as needed.

Note

This modified version of MS medium includes:

- Macro- and micronutrients as described by Murashige and Skoog (1962)
- Vitamins as described by Linsmaier and Skoog (1965).
- Ferric Sodium EDTA in place of Ferrous Sulfate and Disodium EDTA.
- 1.0 mg/L Kinetin.
- 0.3 mg/L IAA.

4.2 **Transfer surface sterilized seedlings to root induction medium** 1h

- Gently remove each seedling from the conical tube (from the rinsing step).
- Place the seedling on the surface of agar plates with root induction medium (see above for the recipe).
- Make sure the seedling, and especially the crown area, is in contact with the root induction medium.
- Seal the petri dish with parafilm.

https://www.youtube.com/embed/TCper5h1qgg

4.3 **Root induction** 1w

- Maintain the seedlings under ~100 µE under a light regime of 16 h light-8 h dark
- New roots will emerge in ~3 days.

Rooted seedlings will be ready for transfer to the 1 L culture vessels ~7-10 days after starting the root induction.



Fig.4- Root induction.

Transfering re-rooted seedlings into culture vessels

5 The seedlings re-rooted in the Petri dishes are now ready to be grown in the large culture vessels.

5.1 **Prepare the plant-growing medium** 2h

The plant medium is prepared as the root induction medium in step 4.1. The only modification is that instead of autoclaving the medium, we will first dissolve the agar and then add the medium to the culturing vessels for autoclaving.

- Autoclave for 15 minutes to dissolve the agar.
- Place 150 mL of the root induction medium (step 4.1) into 1 L culture vessels.
- Close the culture vessels.
- Autoclave for 30 minutes to sterilize the media.

5.2 **Transfer the rooted seedlings into the culture vessels.** 1h

1. Using forceps in the hood, remove the seedling from the petri dish.
2. Place the seedling in the 1 L culture vessel.
3. Gently push the seeds into the media.

4. Grow the plants under a light regime of 16 h light-8 h dark and a light intensity of ~200 µE.
5. The seedlings will form new leaves and roots, and the plantlets will develop new lateral shoots and rhizomes that could be used for in vitro multiplication.



Fig. 5- Re-rooted seedlings growing in the culture chamber.

In vitro propagation of *Sporobolus alterniflorus*

1h

6 1h

Once plants have produced new shoots, we can easily separate each of them from the mother plant and place them in new culturing vessels. The new lateral shoots developed from the main shoot also have a root system. Once separated they will develop into full adult vitroplants in 4-6 weeks. Even shoots as small as 1-2 cm can grow into full vitroplants as long as they have roots. Rhizomes can also be used as explants and they will also develop into complete plants.

At this stage, you can continue growing the vitroplants in the modified MS media from step 4.1 or transfer the plants to a hormone-free medium, MS medium (3% sucrose, pH 5.8, agar 0.7%).

https://www.youtube.com/embed/FFf9bLiZ5Xw

Note

You can transfer the plants into the media when the agar is cool to the touch but not completely settled. This way, it is easier to fully cover the roots in media!

Protocol references

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