# Sterile Culture Techniques for Characterization of Root Exudates

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## Abstract

Creating and maintaining sterile conditions in the root zone while providing optimal plant growth conditions is a challenging task. Microbial activity and stresses resulting from excessive or inadequate air, water, and nutrients can alter root exudation. Therefore, sterile containers must provide optimal water-holding capacity and air-filled porosity while facilitating control of nutrient status. We developed a solid-media plant growth container for use in exudate studies (see Henry et al., Characterization of Root Exudates from Crested Wheatgrass, ASA paper 2001). All components are autoclavable. Containers are filled from bottom to top with a five-layer sand gradient to uniformly distribute water and air. A foam plug seals the shoot/container interface. Sterile, dilute nutrient solution is added through a septum via a sidearm port. A 7-cm drain tube with silanized glass wool wick provides consistent drainage and enables leachate collection for exudate analysis. Seeds are surface sterilized and grown on non-selective agar to confirm sterility prior to transplanting. As plants grow, sterility is periodically verified by plating out samples of both sand media and leachate. Plants grown for one month in these containers exhibited exponential growth and uniform root distribution within each container. Sterility was maintained in many plants for 30 days. We continue to refine our sterile technique.

## Objective

To develop a plant growth container that 1) maintains optimal plant growth in a sterile (aseptic) root-zone environment and 2) enables collection of root exudates.

## Materials and Methods

### Plant Growth Container Components

All components of the plant growth containers (Figure 1) are autoclavable. The containers allow aseptic addition of nutrients and collection of leachates, and eliminate the potential for over watering. Ottawa sand was selected as the growth medium because it is inert, autoclavable, and has relatively good water holding capacity.

The plant growth containers are constructed of 38-mm diameter glass tubing. Each container has a sidearm and septum for aseptic addition of nutrient solution. The growth medium is supported by a 1-hole silicone stopper and a drainage tube fitted with a silanized glass wool wick. A 2-hole silicone stopper (one hole for water to drain and one for air displacement) connects the drainage tube to a removable 40-mL amber vial.

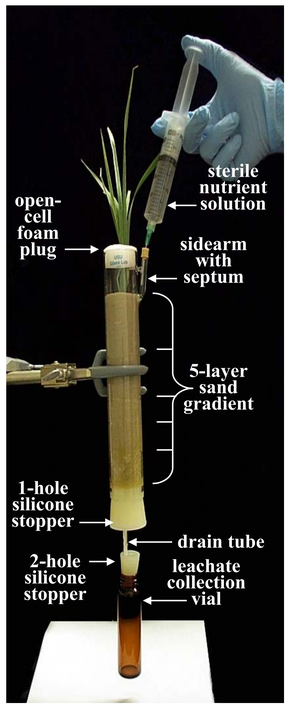


**Figure 1.** Plant growth container.

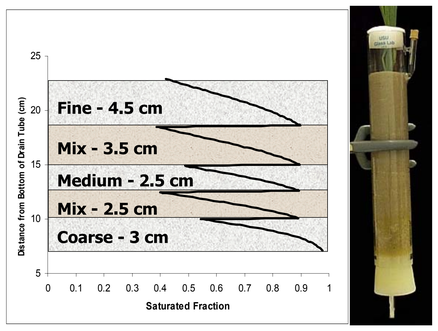


**Figure 2.** Water retention in the plant growth container. The sand gradient keeps all parts of the column between 40% and 90% of saturation.

A plant growth test was conducted in three grain sizes of Ottawa sand to determine the trade-off between water-holding capacity and air-filled porosity. This test led to the use of a 5-layer sand gradient to uniformly distribute water and air from the bottom to the top of the column according to the the van Genuchten (1980) water retention model (Figure 2). The sand layer thicknesses, from bottom to top, are as follows: 3 cm coarse (20-30 grit); 2.5 cm medium/coarse mix (20-40 grit); 2.5 cm medium (30-40 grit); 3.5 cm fine/medium mix (30-50 grit); 4.5 cm fine (40-50 grit).

### Sources of Carbon Contamination

We discovered that several of the container components had organic carbon associated with them: **Ottawa sand** had significant organic contamination. After testing several treatments to remove the organic contamination we determined that heating the sand in a muffle furnace at 550°C for at least 10 hours was most effective at removing the contamination (Figure 3). We were surprised to find that the **glass wool** was also contaminated with organic matter. This contamination can be removed by thoroughly washing the glass wool with deionized water (Figure 4). Silanized glass wool is now being used both for its low organic content and because it will not cause breakdown and adsorption of certain compounds. Silicone **stoppers** are used instead of rubber stoppers to minimize carbon contamination from the stopper (Figure 4).

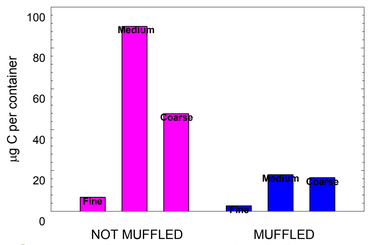


**Figure 3.** Carbon associated with Ottawa sand before muffling (pink) and after muffling (blue).

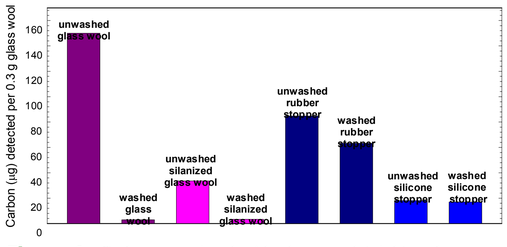


**Figure 4.** Carbon associated with glass wool (pink) and stoppers (blue).

### Container Assembly and Sterilization

Empty glass containers are washed with methanol, rinsed with deionized water, and baked at 170°C for a minimum of two hours. Sand is washed with deionized water and then baked in a muffle furnace at 550°C for at least 10 hours. Silicone stoppers equipped with drain tubes are washed with methanol, rinsed with deionized water, and and baked at 80°C until dry. Stoppers are inserted at the base of each container and individual sand layers are added. The containers are autoclaved twice, 24 hours apart, to effectively kill any bacterial spores that exist in the sand. Rubber septa and 2-hole silicone stoppers are autoclaved once and attached to the autoclaved containers in a laminar flow hood. The containers are placed in boxes designed to shield root zones from light and to allow laminar flow of sterile air past the leachate collection vials during leachate collection (Figure 5).

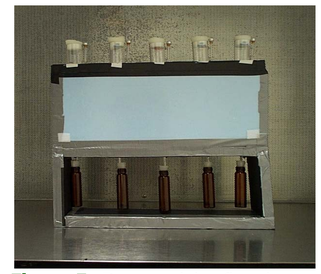

**Figure 5.** Box containing five plant growth containers in laminar flow hood.

### Seed Sterilization

Seed sterilization techniques must eliminate all microorganisms with minimal effects on seed vigor. After several preliminary tests (Figure 6), we found that soaking the seeds in a 20% Clorox solution for one hour sterilizes crested wheatgrass seeds without significantly reducing seedling vigor. Based on our test results we now use a one hour soak in 20% Clorox with 0.1% Tween 80 followed by a deionized water rinse.

**Figure 6.** Culture plate with crested wheatgrass seeds.

### Plant Growth Conditions

For our first trial, sterile plants were grown in a reach-in plant growth chamber (Enconair, Model CG-9) with a photoperiod of 16 hours and a photosynthetic photon flux (PPF) of ~350 µmol $m^{-2}$ $s^{-1}$. Temperatures were set to 25°C during the day and 20°C at night. For our current trial, we have moved to growing our sterile plants in a laminar flow hood equipped with two high pressure sodium lamps (Figure 7). The photoperiod and PPF are similar to the growth chamber. However, temperatures are now 30°C during the day and 25°C at night. Plants are watered with a dilute nutrient solution containing no silica or chelate.

**Figure 7.** Sterile plants in laminar flow hood equipped with HPS lamps.

### Testing for Sterility

Once per week, a sample of either the leachate or the sand from the top surface of each column is plated on 1/10 strength nutrient agar to check for microbial contamination (Figure 8). This dilute medium is necessary for optimum microbial growth since higher concentrations could shock the organisms and impede growth. Plates are incubated at 26°C and are observed for several weeks since some slow growing microorganisms take several weeks to appear. Possible errors in checking for contamination include false positives due to outside contamination while plating, and false negatives due to inadequate cooling after flaming the inoculating loop (thereby killing the contaminants). Additionally, less than 1 % of contaminants are culturable on agar media, so some contamination may go undetected.


**Figure 8.** Plate showing con-

## Results

### Sterility

Sterility was maintained in many plants for 30 days (Figure 9) when the plants were grown in a growth chamber that circulated non-sterile air. In our current trial, the plants are grown within a laminar flow hood (Figure 7).

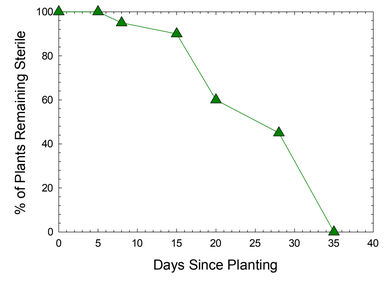


**Figure 9.** Sterility of plants (grown in growth chamber) over course of 35-day study.

### Plant Growth

At harvest, plant roots were evenly distributed from the top to the bottom of the plant growth containers (Figure 10). The plants grew exponentially, as indicated by transpiration data (Figure 11).

**Figure 10.** Harvested crested wheatgrass plants.

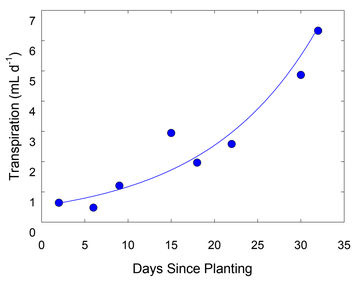


**Figure 11.** Exponential increase in transpiration rate of a representative sterile plant.