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In vitro Conditions for Dark Growth and Analysis of Maize Seedlings

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[Abstract] Maize is an important model organism for understanding plant traits essential for proper growth and germination. One type of growth, skotomorphogenesis, occurs in the absence of light. Seedlings grown in the absence of light exhibit dramatic differences in stem and leaf development compared to light-grown plants. Dark-growth conditions require the use of highly controlled plant growth environments. Here, we provide step-by-step instructions for creating a soilless and dark plant growth environment for maize using half-strength Murashige and Skoog media solidified with agar in clear boxes that are covered in aluminum foil. The benefits of this protocol are that it does not require special dark-growth conditions and the growth media can be easily and uniformly supplemented with hormones or other chemicals.
Keywords: Maize, *In vitro*, Skotomorphogenesis, Seedling, Dark-growth, Mesocotyl

[Background] Soilless media and temperature-controlled environments have been used for over eighty years to generate reproducible plant growth (McClary, 1940; George *et al.*, 2008). Murashige and Skoog (MS) media, containing salts, amino acids and vitamins, is one of the most commonly used plant growth medias (Murashige and Skoog, 1962). MS was originally developed for tobacco but has since been used to grow other plant species including *Arabidopsis* and maize. This growth media does not readily react with phytohormones unlike other soilless media such as peat, perlite and vermiculite (Best *et al.*, 2014 and 2017; Malik *et al.*, 2014). Therefore, hormones can be added to the growth media to compare responses between wild-type and mutant growth.

The rolled paper towel method is the standard for high throughput quantitative analysis of maize seedling germination (Phaneendranath, 1980). Maize kernels are sterilized, and rolled in moist paper towels, then allowed to grow. Because microbes can influence plant growth, this method often includes a fungicide solution in addition to kernel surface sterilization (Pace *et al.*, 2014). Rolled or vertical filter paper can also be used to grow maize seedlings in combination with hormone application (Camp and Wickliff, 1981; Hahn *et al.*, 2008). Other methods for growing maize in soilless environments may use custom-built systems (Singletary and Below, 1989).

Plants grown in the absence of light undergo skotomorphogenesis (Josse and Halliday, 2008). This developmental program causes the stem and leaves of plants to elongate via anisotropic cell expansion toward potential light sources (Cheng *et al.*, 2000; Markelz *et al.*, 2003; Wang *et al.*, 2016). Dark growth can be achieved by light-controlled growth chambers (Wang *et al.*, 2016), or by placing maize seedlings grown in clear plastic boxes with moist cellulose filter paper into light-tight black boxes (Waller and Nick,

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1997). Dark-growth of *Arabidopsis* has been achieved by covering plates in aluminum foil (Alabadí *et al.*, 2004).

We combined a number of these popular methods to create a controlled growth environment for dark-grown maize seedlings. We first sterilized maize kernels from the B73 inbred background (Martínez and Wang, 2009). Next, kernels were grown in soil or on solid MS media in clear plastic (“magenta”) boxes and subjected to either standard (16-h light, 8-h dark) or constant dark conditions using aluminum foil to block light (Alabadí *et al.*, 2004). These boxes are commercially available, relatively inexpensive, maintain sterile conditions, can withstand repeated autoclave cycles, and are easy to cover with foil to block light. However, their use is limited to maize seedlings less than two-weeks old due to their relatively small size. We found that the B73 mesocotyls grown in soil were longer in both standard and dark treatments compared to standard and dark treated seedlings grown on ½ MS media solidified with agar. However, we observed that the mesocotyl lengths of B73 seedlings grown on ½ MS were more consistent. Taken together, we show that the method described here creates a reproducible environment for quantitative analysis of dark-grown maize mesocotyl length. This method could also be used to study maize seedling growth responses to hormones, abiotic stress conditions, or other treatments.

Materials and Reagents

1. 100 µl pipette tips
2. 50 ml conical tube (VWR, catalog number: 10025-698)
3. Magenta Boxes (Fisher Scientific, catalog number: 50-255-175)
4. Magenta couplers (Caisson Laboratories, catalog number: MK5C-20PC)
5. Paper towels
6. Gloves
7. Spray bottle
8. 2 ml centrifuge tubes
9. Aluminum foil
10. Bleach (Sigma-Aldrich, catalog number: 13440, CAS: 7681-52-9)
11. Distilled sterile water
12. 200 Proof ethanol (Sigma-Aldrich, CAS: 64-17-5)
13. MS (Murashige and Skoog Medium) (Fisher Scientific, catalog number: 11434865)
14. MES (2-ethanesulfonic acid) (Sigma-Aldrich, catalog number: M2933, CAS: 4432-31-9)
15. Potassium Hydroxide (KOH, Sigma-Aldrich, catalog number: P5958, CAS: 1310-58-3)
16. Agar (Teknova, catalog number: A7777)
17. 80% ethanol (see Recipes)
18. 50% bleach (see Recipes)
19. ½ MS media (see Recipes)
20. 1 M KOH (see Recipes)
21. 70% ethanol (see Recipes)

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Equipment

1. Magnetic stir bar
2. 100 ml graduated cylinder
3. 500 ml graduated cylinder
4. 2 L beaker
5. 250 ml Erlenmeyer flask
6. 100 µl pipette
7. pH Probe
8. Rocking platform
9. Microwave
10. Autoclave
11. Sterile laminar-flow hood
12. Growth chamber (Percival, model: AR66L)
13. Metric ruler
14. Flatbed scanner

Software

1. FIJI (ImageJ, https://imagej.nih.gov/ij/download.html)
2. Prism6 (GraphPad, https://www.graphpad.com/scientific-software/prism/)

Procedure

A. Seed sterilization
   1. Place 100 seeds in a 250 ml Erlenmeyer flask along with a stir bar.
   2. Add 100 ml of 80% ethanol.
   3. Place flask on stir plate and set the rotation speed to low.
   4. Incubate at room temperature for 3 min.
   5. Decant ethanol.
   6. Add 200 ml of 50% bleach.
   7. Place flask on stir plate at low speed and incubate for 15 min.
   8. Decant bleach and rinse seeds with sterile distilled water in the hood 5 times.
   9. Add 200 ml of 50% bleach.
   10. Place flask on the stir plate at low speed and incubate for 15 min.
   11. Decant bleach and rinse seeds with sterile distilled water in the hood 5 times.
   12. In the hood, add 5 ml of sterile distilled water and cover with foil.
   13. Incubate on a rocking platform so that all seeds are gently moving for 24 h.
   14. In the hood, rinse seeds with 100 ml of sterile distilled water.

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15. Decant water into a liquid waste container and repeat the rinse 4 more times.
16. Decant final rinse and remove stir bar from the Erlenmeyer flask.
17. In the hood, scatter seeds on sterile media from the Erlenmeyer flask by pouring.

B. Media preparation and seed addition to boxes

1. Pour 50 ml of molten ½ MS media (Recipe 3) into each bottom (no adapter) magenta box of 20 total boxes (Figure 1B). This step and the following step do not need to be done in the hood.
2. Cover both top (with adapter pre-attached) and bottom boxes with foil.
3. Autoclave on liquid cycle for 30 min to sterilize media and boxes.
4. Steps B5-B9 are done in the hood.
5. Allow media to solidify in the bottom containers, as shown in Figure 1A.
6. Remove foil from bottom container and add 5 sterilized seeds, as shown in Figure 1B.
7. Remove foil from top container (with adaptor).
8. Securely place top container onto bottom container with media and seeds as shown in Figure 1C.
9. Repeat the two previous steps with all containers.
10. Wrap closed containers with foil to block light.
11. Incubate containers in a growth chamber set to 24 °C for ten days.

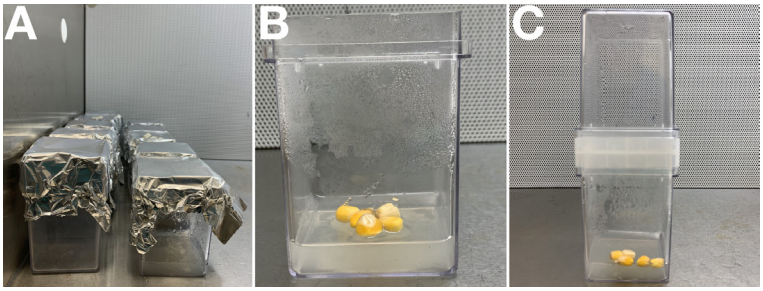

Figure 1. Container additions and closing. A. Four top and bottom boxes allowed to solidify in sterile hood. B. Five seeds added to the bottom box containing solid media. C. Closed container with five seeds.

C. Scanning seedlings

1. Number each container 1-20.
2. Assign each seedling a letter A-E.
3. Name each image file with the current date, treatment, and container number.
4. Place a metric ruler face down on the scanner bed.
5. Place each seedling with the aerial tissue facing the scanning surface. Next to each seedling place a letter designation token (piece of paper) face down as shown in Figure 2A.
6. Scan into a pre-named folder.
7. Place a sliver, approximately 0.1 g, of each shoot into a corresponding pre-labeled 2 ml centrifuge tube for later DNA extraction.

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8. Repeat above steps for all containers.

D. Measuring seedling mesocotyls

1. Open scanned file in ImageJ or FIJI.
2. Set scale (scales differ per scanner). If scale is unknown, set scale for everything to 1 and use the straight-line tool to measure 1 cm on the metric ruler in your scanned image. Set your scale to the pixels measured in 1 cm from the line that you drew.
3. Use the segmented line feature to trace the mesocotyl of each seedling, measuring each one as you go as shown in Figure 2B.
4. Copy and paste measurements from ImageJ to a pre-labeled google sheet.

Figure 2. Scanning and measuring. A. Each seedling is placed with the aerial tissue facing the scanning surface with a designated token near the seed. B. Measuring mesocotyl length using the segmented line measurement tool in FIJI (ImageJ).

5. Assign traced measurements to their corresponding seedlings (Video 1).

Video 1. Movie showing how to measure mesocotyl lengths using FIJI

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E. Analyzing seedling mesocotyl length (Figure 3)

1. Copy and paste measurements by group (genotype, organ) into graphing and statistical analysis software (Prism6, GraphPad).
2. Select the method of analysis that is most appropriate (Mann-Whitney Test).

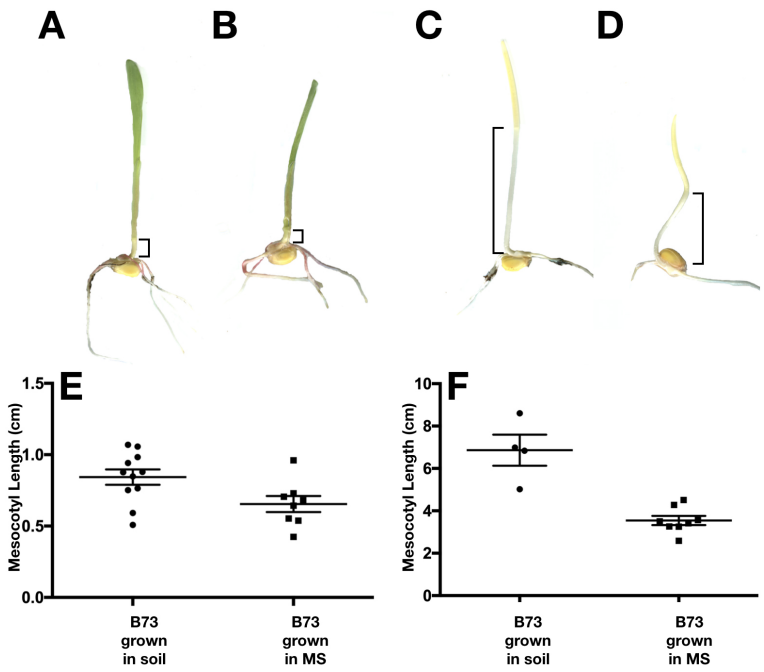

Figure 3. Wild-type seedlings grown in soil or ½ MS media in either light or dark conditions. Comparison of mesocotyl length in soil and ½ MS media, and grown in standard conditions (16-h light and 8-h dark cycling) and dark conditions for ten days. Brackets indicate location of measurement for mesocotyl length. A. B73 seedling grown in soil with a mesocotyl length of 0.87 cm in standard conditions. B. Standard light/dark cycling of B73 grown on ½ MS media with a mesocotyl length of 0.96 cm. C. Dark-grown seedlings grown in soil with mesocotyl length of 5.02 cm. D. Dark-grown seedlings grown on ½ MS media with mesocotyl length of 3.56 cm. E. Mesocotyl length for seedlings grown under light conditions in soil or ½ MS media. F. Mesocotyl length measurements for seedlings grown under dark conditions in soil or ½ MS media.

Notes

1. Always wear gloves and a lab coat when handling ethanol and bleach solutions.
2. Avoid tearing the foil during wrapping to prevent light contamination (see Figure 4A).
3. Thoroughly decontaminate sterile hood prior to the start of this protocol (see Figure 4B).
4. Carefully close the boxes to reduce contaminant introduction.
5. 5 ml is the optimum amount of imbibing water during overnight incubation. Excess imbibing water reduces germination rates (see Figure 4C).

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6. MES is added to the media to stabilize the pH during the autoclaving process.
7. Remove the magnetic spin-bar from the beaker prior to heating media.
8. Ensure that all closed containers are face up (seeds are on the sterile media) prior to wrapping with foil.
9. Use caution while melting agar in the microwave. Multiple cycles of heating and cooling will be required to melt the ½ MS media. When the solution begins to boil, stop, mix with a stir bar, then remove the stir bar, and allow it to cool. Finally, resume heating in the microwave until it is melted.

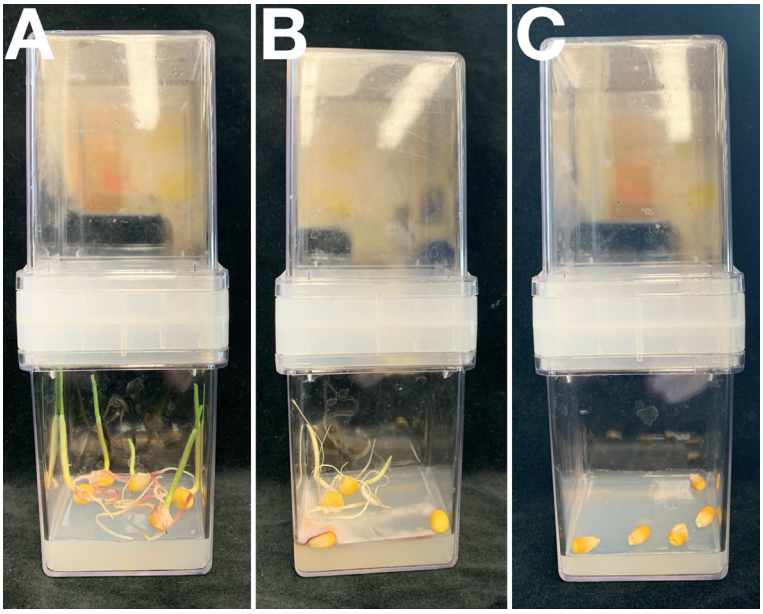

Figure 4. Examples of common problems. A. Green leaves on seedlings indicate light contamination. B. Fungal contamination can occur by lack of aseptic technique or due to contamination not removed by surface sterilization. C. Lack of germination may be due to growing conditions, too much water used during overnight incubation or excess bleach not removed during wash steps.

Recipes

1. 80% ethanol
   Note: May be made ahead of time and stored at room temperature for up to 1 year.
   Add 80 ml of 100% ethanol to a 100 ml graduated cylinder and raise the volume to 100 ml with distilled water
2. 50% bleach
   Note: May be made ahead of time and stored at room temperature for up to 6 months.
   Add 200 ml of 100% bleach to a 500 ml graduated cylinder and raise the volume to 400 ml with distilled water
3. ½ MS media (1,000 ml)

Note: Make fresh each time.

a. Add 1,000 ml of distilled water to a 2 L beaker with a magnetic spin bar
b. Place beaker on stir plate
c. Place pH probe in solution
d. Add 2.2 g of MS
e. Add 0.5 g of MES
f. Raise pH of media to 5.7 with 1 M KOH
g. Remove pH probe from beaker
h. Add 8 g of agar (0.8%)
i. Melt media in microwave

4. 1 M KOH

   Note: May be made ahead of time and stored at room temperature for up to 6 months.

   a. Add 50 ml of distilled water and a stir bar to a 250 ml beaker, then add 5.61 g of KOH
   b. Raise the volume to 100 ml in a graduated cylinder once all of the KOH has dissolved

5. 70% ethanol

   Note: May be made ahead of time and stored at room temperature for up to 1 year.

   Add 350 ml of 100% ethanol to a 500 ml graduated cylinder and raise the volume to 500 ml with distilled water

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Competing interests

All authors declare no competing interests.

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