**ADVANCED BIOTECHNOLOGY** LESSON 2

# Plant cloning

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| **Focus question** | How might we create a sterile growing environment? How important is a sterile environment? How is a plant clone produced? |
| **Learning target** | Students will maintain a sterile growing environment. Students will propagate many plants of the same genetic background. |
| **Vocabulary** | Plant tissue, tissue culture, sterile, aseptic technique, explant, nutrient medium |

This lesson was adapted from Plant Tissue Culture.
apsnet.org/edcenter/K-12/TeachersGuide/PlantBiotechnology/Documents/PlantTissueCulture.pdf

## LS3: Heredity: Inheritance and Variation of Traits

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| **Performance expectation** HS-LS1-4 | **Classroom connection:** Students will attempt to grow new plant tissue from existing plants. This will model cloning and the role of cell division and differentiation. |

## Science and engineering practices

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| **Developing and Using Models** | **Classroom connection:** Students will choose a plant “model” to use as a tissue sample and clone it by growing it in culture. |

## Disciplinary core ideas

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| **LS1.B: Growth and Development of Organisms** | **Classroom connection:** Students will choose a plant tissue, research the media needed to grow that plant tissue in culture, prepare the media, sterilize the plant tissue, and grow the plant in the culture media. |

## Cross-cutting concepts

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| **Systems and System Models** | **Classroom connection:** Students will model a sterile environment, determine the importance of the sterile environment when plants are trying to grow, and examine the results of a less-than-sterile environment. |

## Background

Watch *Tissue Culture Propagation: Class 101* before beginning: **youtu.be/qDOGrEhUe8A**. It may be helpful to share this video with students as well. Advanced students may be able to help with preparation of agar. There are many parts of this lab, but all require sterile technique. On the student handout, the procedure for *Plant preparation* and *Transfer of plant material* to tissue culture medium are included along with the materials needed for that procedure.

Plant research often involves growing new plants in a controlled environment. These may be plants that we have genetically altered in some way or multiple copies of cloned plants. This can be accomplished through the tissue culture of small tissue pieces from the plant of interest. These small pieces may come from a single mother plant or they may be the result of a genetic transformation of single plant cells, which are then encouraged to grow and to ultimately develop into a whole plant. Tissue culture techniques are often used for commercial production of plants as well as for plant research.

Tissue culture involves the use of small pieces of plant tissue (explants), which are cultured in a nutrient medium under sterile conditions. Using the appropriate growing conditions for the explant type, plants can be induced to rapidly produce new shoots and new roots. These plantlets can also be divided, usually at the shoot stage, to produce large numbers of new plantlets. The new plants can then be placed in soil and grown in the normal manner.

*Note:* Many types of plants are suitable for use in the classroom. Cauliflower florets particularly give excellent results since they can be grown into a complete plant in the basic tissue culture media, without the need for additional growth or root hormones. Green shoots are generally observable within three weeks, and roots develop within six weeks. If other plants are used, different materials will need to be added to the growth media to encourage root growth.

The most important part of this activity, however, is to maintain as sterile an environment as possible. Even one fungal spore or bacterial cell that comes into contact with the growth media will rapidly reproduce and soon completely overwhelm the small plant.

## Prior knowledge

Students should be familiar with mitosis and may be familiar with cloning. The process of tissue culturing eventually results in differentiation of plant tissue into roots and shoots. The plant is very vulnerable to any type of fungal growth during this time, however. Discuss what is meant by sterile environment to help students understand the myriad of organisms that can contaminate the growth environment they are attempting to create.

## Materials

- 1 Vial of Murashige Skoog (MS) media
- 1 L sterile distilled water
- 9 g of agar/L
- 30 g sucrose/L
- The first container should have BAP added at the rate of 2.0mg/l. The second container should have the NAA hormone added at the rate of 0.1 mg/L. To do this, it is necessary to make concentrated solutions of both BAP (2.0mg/ml) and NAA (1.0mg/ml) and filter sterilize them (see: **youtu.be/vcxy6FpfSuw** for a demonstration). Add 1ml of the concentrated BAP stock or 100µl of the NAA concentrated stock to each 1 liter of media that you prepare.
- 1.5 L or 2 L flask in which to prepare the growth medium
- Sterile culture tubes with lids
- Small amounts of 1M NaOH and 1M HCl to adjust the pH of the media
- pH paper (5.0–7.0 by tenths)
- Aluminum foil
- Glass aquarium or plastic oven bag or Laminar flow hood

- Plastic sheet to cover the top of the aquarium (if preferred over plastic bag)
- Adhesive tape
- Hot plate with stir bar
- 70% alcohol (ethanol) in a spray bottle
- Sterilized forceps or tweezers
- Sterile petri dishes (for cutting)
- Gloves
- Sterile cutting equipment such as a scalpel blade, razor blade or cork borer
- 2 gallons of sterile distilled water
- Cauliflower florets (or other plant tissue or fresh leaves)
- Lidded containers (or beakers) to wash the plant material
- Detergent-water mixture: 1mL detergent per liter of water
- Sterilizing solution: 10% bleach solution (250 mL household bleach + 2250 ml water)
- 2 or 3 beakers or jars for sterile water
- A well-lit area away from direct sunlight or use full-spectrum grow-light

## Teacher preparation

### Agar preparation (about 1 hour)

These steps will make 500 mL of growth medium, which is enough to prepare about 65 growing tubes.

1. Add 400 mL distilled water to a 1 L flask. Dissolve the Murashige-Skoog (MS) powder in the water. Stir the water continuously while adding the salt mixture using a magnetic spin bar in the flask while on the hot plate.
2. Add 15 g of sugar and continue to stir to dissolve. Adjust pH to 5.8 using drops of 1M NaOH or 1M HCl as necessary while gently stirring.
3. Add distilled water to make the total volume up to 500 ml.
4. Mass 4.5 grams of agar and add it to the MS solution.
5. Cover the flask with foil and heat on high and stir on the hot plate until the liquid is just boiling. Agar will boil over quickly.
6. Simmer the liquid for 30 minutes on low heat and let cool to touch (to about 50oC).
7. Pour the still-warm medium into the culture tubes until one-third full. Cover immediately to cool.
8. Store culture tubes in sets of 6–8 in test tube racks.
9. Place the culture tubes in an autoclave to sterilize.

### Sterilize transfer chamber and equipment preparation

(Students could be assigned to make their own sterile chambers if desired, or there can be one for class use that will need to be closely monitored as groups cycle through.)

If a laminar flow hood is available, this should be used to maintain a sterile environment. If not, follow the steps below to create a sterile working environment for the transfer of materials to the growth media.

1. A classroom transfer chamber can be made from a clean glass aquarium turned on its side. Scrub the aquarium thoroughly with a 10% bleach solution in a chemical hood if possible, making sure that you wear gloves and do not inhale the fumes. Rinse with sterile distilled water, turn upside down on a clean counter or paper towels and allow to dry.
2. Cut holes in a clean plastic sheet to allow arms to reach into the chamber and reinforce the cut edges with tape if necessary. Tape the clean plastic sheet over the open side of the aquarium making sure that the armholes are located at a convenient height. Plastic sleeves could also be fitted to these holes if you wish to make it easier to prevent the entry of airborne spores into the chamber. The finished aquarium chamber can be sterilized by spraying with 10% bleach solution just prior to each use and drying with sterile paper towel.

3. Alternatively, a plastic oven bag can be used. Plastic bags are sterile until opened since they are heated when formed. The bag can be sterilized by spraying with 10% bleach solution just prior to each use and drying with a sterile paper towel.
4. Wrap the forceps, scalpels, razor blades, and/or cork borer, paper towels, and gloves (rubber or surgical) in aluminum foil, seal with tape and sterilize by placing in an oven at 350°F for 15 minutes. You can wrap each item separately or put together a "kit" so that each student will have their own sterile equipment to use.
5. Alternatively, the forceps and blades can be sterilized by dipping in 10% bleach solution and then rinsing in sterile water. If you choose to dip in bleach and rinse in sterile water, it is best if fresh solutions are available for each student group, since the water can easily be contaminated if care is not taken. These liquid containers should only be opened when they are inside of the sterile chamber.

## Plant preparation

(Assign to students for their own groups to use.)
You will be sterilizing the plant material to remove any bacteria or fungal spores that are present. The aim is to kill all microorganisms, but at the same time not cause any adverse damage to the plant material.

1. Cut cauliflower into small sections of florets about 1 cm across on a sterile surface within the sterile transfer chamber. If using plant leaves, cut into disks with a sterilized cork borer.
2. Wash the prepared plant material in a detergent-water mixture for about 20 minutes. This will help remove fungi etc., and the detergent will help wet the material and remove air bubbles that may be trapped between tiny hairs on a plant.
3. Transfer the washed plant material to the sterilizing bleach solution. Shake the mixture for 1 minute and then leave to soak for 10–20 minutes. Carefully pour off the bleach solution, using the lid to keep the plant tissue from coming out, then carefully cap the container.

*Note 1: At this point, the tissue is considered sterile. All subsequent rinses should be done with sterile water and all manipulations of the tissue performed with sterile instruments and supplies. Open one container at a time and never leave the lid off of any container longer than necessary.

*Teacher Note: Many students will not fully appreciate the importance of carefully sterilizing explants and there will be some cultures that become infected with bacterial or fungal growth. If you do not wish to emphasize this aspect of the laboratory, students can be provided with plant materials that the instructor has already sterilized prior to use by the class.

## Transfer of plant material to tissue culture medium

Use sterile gloves and equipment for all of these steps.

1. Spray the outside surfaces of the containers, the capped tubes, and the aluminum-wrapped supplies with 70% alcohol before moving them into the chamber.
2. Place the plant material still in the bleach sterilizing container, the containers of sterile water, the sterilized forceps and blades, some sterile paper towels to use as a cutting surface, and enough tubes containing sterile medium into the sterile area.
3. Spray the gloves with a 70% alcohol solution and rub hands together to spread the alcohol just prior to placing hands into the chamber. Once gloves are on and sprayed, they must not touch anything that is outside of the sterile chamber.
4. Carefully open the container with the plant material and pour in enough sterile water to half-fill the container. Replace the lid and gently shake the container to wash tissue pieces (explants) thoroughly for 2–3 minutes to remove the bleach. Then let sit for 15 minutes. Pour off the water.
5. Remove the sterilized and washed plant material from the container and place on the paper towel or sterile petri dish. Cut the cauliflower into smaller pieces about 2 to 3 mm across. Be

sure to avoid any tissue that has been damaged by the sterilizing solution, which is apparent by its pale color. If using a disk of plant leaf, no additional cutting is necessary.

6. Take a prepared section of plant material in sterile forceps and place into the medium in the culture tube. Cauliflower pieces should be partly submerged in the medium, flower bud facing up. If using another plant leaf, be sure the disk is in contact with the agar.
7. Replace the lid on the culture tube.

## Growing the plants

1. Place plant sections in a well-lit area of the classroom although not in direct sunlight. The shoots will grow more quickly if the explants are placed under fluorescent or grow-lights to provide at least 12 hours of light per day. The aquarium can be used as a growth chamber with the lighting about 8–10" overhead. This will also help maintain a more regular and warm temperature. Ensure that the temperature does not go over 82.4°F. New shoots should develop within 2 weeks and should be well advanced in 3 to 4 weeks. Check the tubes daily and discard any that show signs of infection (before discarding, add bleach into the tube). Roots can appear within 6 weeks on cauliflowers.
2. Working inside the sterile aquarium chamber, remove the lid from the culture dish. There will usually be several shoots that have arisen from each explant. These shoots should be carefully separated by gently removing the whole explant from the media with sterile forceps and then separating the shoots by gently pulling them apart using two pairs of forceps. Each shoot should then be placed into a tube of rooting media and the bottom of the shoot pushed into the media so that good contact is made. The cap is replaced and the shoots are then allowed to grow as in step 1 until roots are formed, usually within 2–3 weeks.

## Differentiation

Other ways to connect with students with various needs:

- **Local community:** Students may do a search to see if there is a plant genetics lab nearby. Searching for "agriculture research and development" may give a list of facilities in your area.
- **Students with special needs (language/reading/auditory/visual):** This lesson includes kinesthetic activities as well as reading and following directions. Students in cooperative groups can rotate tasks and utilize all students' strengths.
- **Extra support:** Watch *Tissue Culture Propagation: Class 101:* `youtu.be/qDOGrEhUe8A`
- **Extensions:** Students may want to try additional plant cuttings. Students will need to research what growth media/nutrients are required for different plants to grow.

## Assessments

### Rubric for assessment

| Skill | Developing | Satisfactory | Exemplary |
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| Use a model to illustrate the role of cellular division (mitosis) and differentiation in producing and maintaining complex organisms. | Student used a plant model of his/her choice to show cellular division through plant tissue culture. | Student used a plant model to show cellular division through plant tissue culture. Student can explain how the process of mitosis leads to plant tissue culture. | Student used a plant model to show cellular division through plant tissue culture. Student can explain how the process of mitosis leads to plant tissue culture. Student researched additional nutrients to use with specific plant tissue. |
|  | Student used sterile technique. | Student used sterile technique and the explants began to grow. | Student used sterile technique and helped to prepare additional materials to test. |

### Rubric for self-assessment

| Skill | Yes | No | Unsure |
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| It was easy to use sterile technique to sterilize and transfer explants to culture tubes. |  |  |  |
| The explants grew into healthy plants. |  |  |  |