

Handling *Arabidopsis* plants and seeds

Methods used by the Arabidopsis Biological Resource Center (ABRC)

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The methods used by the ABRC for handling *Arabidopsis thaliana* (*Arabidopsis*) plants and seeds are outlined below. These procedures are designed to generate healthy plants that give maximum set of pure seeds and to preserve these in the safest and most convenient manner. The plant and seed management protocols are given in the chronological order in which they would normally be utilized. Many other approaches may be equally as good, especially in specific experimental situations.

1. Growth of plants

Proper handling and maintenance of *Arabidopsis* plants is a prerequisite for accurate and reproducible research, it also enables a high rate of seed production. *Arabidopsis* can be grown in a variety of locations, growth media and environmental conditions. Environmental settings include growth rooms, growth chambers, greenhouses, lighted shelves and outdoors. Peat moss-based mixes, commercial greenhouse mixes, relatively inert media watered with nutrient solutions, and defined agar media can all be employed as plant substrates. Our focus will be on growth of plants on agar and soil in growth chambers and greenhouses.

1.1. Growth of plants in sterile conditions on solid media

It is necessary to grow *Arabidopsis* in sterile conditions on solid media for specific experimental settings such as selection of drug resistant and transformed plants, examination of early root and shoot phenotypes, identification of homozygous lethal mutants, etc. Liquid bleach sterilization, described here, is a practical method to sterilize a few seed lines at a time. Larger numbers of lines can be sterilized easily and with less manipulation using chlorine gas. Chlorine gas can also be utilized for seeds infested with powdery mildew or other fungal diseases. Various containers such as sterile plastic Petri plates (10 cm or 15 cm diameter), Magenta[®] boxes, or culture tubes are used, depending on the purpose of the

experiment. Below we describe the use of the most commonly employed medium for sterile growth conditions in Petri plates (1x MS agar media). Adaptation to other sterile formats is straightforward, and most experimental additives can be easily incorporated in the preparation.

1. Add 4.31 g of Murashige and Skoog (MS) basal salt mixture and 0.5 g of 2-(N-Morpholino) ethanesulfonic acid (MES) to a beaker containing 0.8 L of distilled water and stir to dissolve. Add distilled water to final volume of 1 L. Check and adjust pH to 5.7 using 1M KOH.
2. Divide the media into two 1 L glass bottles, 500 mL in each. Add 5 g of agar granulated per bottle. Keep the lid loose.
3. Autoclave for 20 min at 121°C, 15 psi with a magnetic stirring device in the bottle.
4. Place the bottles on a stir plate at low speed, and allow the agar medium to cool to 45-50°C (until the container can be held with bare hands).
5. Starting from this step, perform all the steps in sterile conditions in a laminar flow hood. Add (optional) 1-2% sucrose and 1 mL Gamborg's Vitamin Solution, stirring to evenly dissolve. Optional sucrose and vitamins should be added after autoclaving and only after the agar media cools, because vitamins are thermo-labile and 15-25% of the sucrose may be hydrolyzed to glucose and fructose at elevated temperatures. Plants grow more vigorously and quickly on media containing 1-2% of sucrose, however, fungal and bacterial contamination must be rigorously avoided by seed sterilization. Note that germination of some mutants might be delayed on sucrose-containing media.
6. Label the bottom of Petri plates with identification number or name, including the date.
7. Pour enough media into plates to cover approximately half of the depth of the plate.
8. Allow the plates to cool at room temperature for about an hour to allow the agar to solidify. If the plates are not to be used immediately, wrap them in plastic and store at 4°C (refrigerator temperature). Covered plates, boxes, or tubes with solidified agar can be stored for several weeks at 4°C in a container that prevents desiccation.
9. Surface sterilize seeds in microcentrifuge tubes by soaking for 20 min in 50% household bleach with the addition of 0.05% Tween[®] 20 detergent.
10. Remove all bleach residue by rinsing 5-7 times with sterile distilled water.
11. For planting of individual seeds at low density, adhere one seed to the tip of a pipette using suction, then release seed onto the agar in desired location. For planting seeds at higher densities, mix seeds in sterile distilled water (or 0.1% cooled top agar), pour onto plate, and immediately swirl to achieve even distribution. Use a sterile pipet tip to adjust the distribution and remove excess water. Allow the water or top agar to dry slightly before placing lid onto plate.
12. Seal with Micropore paper tape to prevent desiccation, while allowing slight aeration.
13. Place the plates at 4°C for 3 days. This cold treatment, also called stratification, will improve the rate and synchrony of germination. The use of an extended cold treatment of approximately 7 days is especially important for freshly harvested seeds, which have more pronounced dormancy. An extended cold treatment is also necessary for certain natural accessions (e.g., Dobra-1, Don-0, Altai-5, Anz-0, Cen-0, WestKar-4). Cold treatment of dry seeds is usually not effective in breaking dormancy. Instead of stratification on plates, seeds suspended in sterile water can also be stratified prior to planting on agar or soil surface.
14. Transfer the plates to the growth environment. Illumination of 120-150 $\mu\text{mol}/\text{m}^2 \text{sec}$ continuous light and a temperature of 22-23°C are suitable growth conditions.

1.2. Growth of plants in sterile conditions in liquid media

Seedlings of *Arabidopsis* can also be grown in liquid growth media. This method provides large amounts of plant tissue suitable for proteomics and metabolomics, or any study that requires a larger amount of starting material. Liquid culture growth is also widely used for high-throughput genomic studies. In this case, growth protocols are adapted to 96 deep-well plates (or other formats) with the MS media supplemented by gibberellic acid.

1. Prepare MS media, as described in subheading 1.1. Do not add agar.
2. After the media has been autoclaved and cooled to room temperature, distribute 75-100 mL MS media into previously sterilized 250 mL Erlenmeyer flasks in a laminar flow hood.
3. Add bleach- or chlorine gas-sterilized seeds to the media (add up to 10 μL of seeds to each flask, which corresponds to approximately 250 seeds).
4. Grow seedlings under continuous light (120-150 $\mu\text{mol}/\text{m}^2 \text{ sec}$) with gentle rotation in an orbital shaker at 120 rpm for up to two weeks.
5. Remove seedlings from the flask. Growth of more than 200-250 seedlings for more than two weeks may result in difficulty removing plant material from the flask.
6. Remove excess media from the seedlings using filter paper. Plant material is now ready for downstream applications.

1.3. Planting seeds on soil

Diverse mixtures and media can be utilized for growing *Arabidopsis*. The term “soil” will be used here for any mix or media utilized for non-sterile growth of plants in pots or similar containers. Commercial potting mixes are popular due to their convenience and reliability. Potting media often employ peat moss for moisture retention and perlite for aeration. Soil mixes such as Sunshine[®] LC1 support healthy *Arabidopsis* growth and include a starter nutrient charge, so that fertilization is not necessary in early growth phases. Soil can be autoclaved to eliminate pests, but this is usually not necessary. Seeds can be planted by various methods: square pots with a diameter of approximately 5.5 cm can be used to grow one plant, 11 cm diameter pots are suitable for growing up to 60 plants, rectangular flats that are 26 cm x 53 cm can accommodate as many as 200-600 plants grown to maturity. Another option especially suitable for genomic studies is 96-well insets. Higher densities, approximately 3000 plants per 30 cm^2 , can be used if plants are harvested at early stages. Preparation of soil for planting in pots can be accomplished as follows:

1. Place soil in a clean container. Add fertilizer in slow release pellets, e.g., Osmocote[®] 14-14-14 (14% nitrogen, 14% phosphate, 14% potassium), feeding up to 3 months from planting. Apply in amounts according to the label. Alternatively, nutrient solution can be used to wet the soil. Wet thoroughly with tap water and mix well with trowel, large spoon or hands.
2. Label plastic pots with holes in the bottom or plastic flats/trays with the stock number or name and date of planting. Always use clean growth supplies, especially new pots and trays to avoid pest contamination.
3. Place soil loosely in pots or other containers using a trowel or large spoon and level, without compressing, to generate a uniform and soft bed. Pots are then ready for planting. Prepared pots can be stored in covered trays at 4°C for several days before planting, although pot preparation and planting should be conducted on the same day if possible.

4. When planting many seeds in a pot, scatter them carefully from a folded piece of 70 mm filter or other paper; distribute them evenly onto the surface of the soil. When planting individual seeds, adhere one seed to the tip of a pipette using suction, then release onto the soil. Various methods can be employed to plant seeds. The density of plants varies with genetic circumstances and purpose of the planting. High yields are achieved with 10-20 plants per 11cm diameter pot. Generally, low densities increase the yield/plant and are suitable for pure lines. High densities reduce the yield/plant, but are useful when it is necessary to maintain the genetic representation in segregating populations. Planted seeds should not be covered with additional soil, since *Arabidopsis* seeds require light for germination.
5. Place pot(s) in a tray, flat or other container.
6. Cover with a plastic dome or with clear plastic wrap taped to the container. The plastic wrap should not be allowed to contact the soil surface and should be perforated to provide aeration. If clear plastic domes are used, they should not be tightly sealed.
7. Place pots at 4°C for 3 days. Most widely used lines have moderate dormancy, and cold treatment, also called stratification, may not be required for germination when planting older seeds of these lines. However, a cold treatment at 4°C for 3 days will improve the rate and synchrony of germination. The use of an extended cold treatment of approximately 7 days is especially important for freshly harvested seeds, which have more pronounced dormancy. An extended cold treatment is also necessary for certain natural accessions (e.g., Dobra-1, Don-0, Altai-5, Anz-0, Cen-0, WestKar-4). Cold treatment of dry seeds is usually not effective in breaking dormancy.
8. Transfer pots into the growth area.
9. Remove plastic dome or wrap for growth in the greenhouse, but leave them on until germinated seedlings are visible for plants grown in a growth chamber.

2. Growth conditions

In general, the growth and development of *Arabidopsis*, including flowering time, is influenced by a number of environmental conditions in addition to the genetic background. Seeds of most lines germinate 3 to 5 days after planting under continuous light, 23°C, adequate watering and good nutrition. Plants produce their first flowers within 4-5 weeks, and seeds can be harvested 8-10 weeks after planting. High quality seeds can be produced if light, temperature and watering are carefully controlled.

You should be aware however, that most of the commonly used growth environmental conditions, particularly in greenhouses, may not be similar to the ones in the native habitats of some natural accessions. This is especially important for interpreting phenotypic differences of traits that are known to be strongly influenced by the natural habitat. Therefore, the protocols described here should be taken only as a guide for the experimental setup and design.

Light

For vigorous plant growth, the optimum light intensity is 120-150 $\mu\text{mol}/\text{m}^2 \text{ sec}$. Higher intensities may result in death of some seedlings, but are tolerated by older plants; purpling of leaves is the first symptom of high light stress. Very low light intensities may result in weak and chlorotic plants. *Arabidopsis* is a facultative long-day plant. Plants flower rapidly under continuous light or long day (>12 h) photoperiods, while under short days (<12 h) flowering is

delayed, favoring vegetative growth. Plants grow well under a cycle of 16 h light/8 h dark, or under continuous light. Various light sources can be used for optimal plant growth, such as cool-white fluorescent bulbs, incandescent bulbs, very high output (VHO) lamps, high-intensity discharge (HID) lamps and shaded sun light. Cool-white fluorescent bulbs, supplemented by incandescent lighting are recommended in growth chambers or growth rooms. HID lamps of 400-1000 watts are conventional in greenhouses in temperate climates to supplement the sunlight or prolong the natural photoperiod.

Temperature

The optimum temperature is 22-23°C. The temperature range for *Arabidopsis* growth is 16-25°C. Lower temperatures are permissible, but higher temperatures are not recommended, especially for germination through early rosette development. Temperatures above 28°C are better tolerated by more mature plants (past early rosette stage). In general, high temperatures result in a reduced number of leaves, flowers and seeds. At lower temperatures, growth is slow, favoring the vegetative phase, and flowering is delayed. Some winter-annual natural accessions require a period of cold to initiate flowering, a process known as vernalization (e.g., Galdo-1, Monte-1, Cit-0, Dog-4, Istisu-1, Valsi-1, Mir-0, Tamm-2). Young rosettes (2-4 weeks old) of late flowering accessions should be placed at 4°C for 4-7 weeks to accelerate flowering.

Water

Water requirement is strongly influenced by relative humidity. Plants tolerate low (20-30%) relative humidity well, but depletion of soil moisture may occur in these conditions. Plant sterility may result from very high (>90%) relative humidity. Mild humidity (50-60%) is considered optimal for plant growth, however low humidity (<50%) is recommended for silique maturation.

The following growth practices are useful for handling plants in any growth context (greenhouse, growth chamber or growth room).

1. Add water to trays containing pots with perforated bottoms.
2. Maintain approximately 2 cm of water around base of pots during germination, to avoid any soil drying before the first true leaves begin expanding.
3. Reduce the watering frequency to as low as once or twice per week as needed after plants have developed true leaves and until the plants flower, to avoid water stress but allow proper drainage of the soil. Plants should not be over-watered to avoid development of algae, fungi, fungus gnat larvae and other pests who thrive on overly wet soil. Algae can be manually scraped off and the soil allowed to dry.
4. Water daily during silique filling stage for good seed production. The water requirement of plants increases dramatically during this stage.
5. Keep plants spaced apart with good air circulation to prevent the incidence of powdery mildew.
6. Place several yellow or blue sticky cards (e.g., Pest TrapTM) in the growth area to monitor insect populations. An important aspect of insect control is detection and identification before populations multiply. These traps are vital in this regard for catching winged insects. Inspect cards and plants daily for pests. Always identify a pest before embarking on

treatment. Change cards periodically to better judge the pest populations, and especially after a pesticide application.

7. Prevent the introduction and spread of pests, which can be transported to the growth area via the soil, seeds, plants, or by humans. Wear a lab coat especially assigned to the growth area, since insects and pathogens can readily be transported on clothing. Plan to have plants of similar age in the growth area, since mature plants are more susceptible to pests than very young plants. Any person who has been in infested growth areas should subsequently abstain from entering non-infested areas; when entering multiple areas, entries should be from the cleanest to the more infested. Keep the area clean and regularly sweep the floors and/or shelves to eliminate or reduce potential sources of pest outbreaks. Mature and dry plants should be harvested and old soil and non-viable dry plant debris should be discarded immediately.
8. Avoid infestation of pests like thrips, aphids, fungus gnats, white flies and spider mites by spraying plants with a preventive mixture of Enstar[®] II and Conserve[®] SC. Insecticide mixture is prepared by adding 1.2 mL of each to 12 L of water. This mix can be sprayed lightly on rosettes prior to bolting stage, before placement of any isolation devices (see subheading 4.1.). Marathon[®] 1G, a granular systemic insecticide, can also be applied as directed by the label to control aphids, fungus gnat larvae, white flies, psyllids and thrips. Marathon[®] 1G is effective as a preventive or as treatment following infestation. It can be applied to the soil surface or included in sub-irrigation watering regime, which reduces damage to the plants. Preventive application of pesticides, if local regulations allow this, can be efficient to avert heavy use of chemicals after infestations have developed. Rotate pesticides, if possible.
9. Note that the use of brand names does not constitute an endorsement of product nor does it imply that other approaches may be necessarily inferior. The chemicals mentioned are for information only. Also, when these or any other pesticides are employed, check the label instructions of the manufacturer before purchase or application for registered usages of the product and recommended application rates and frequencies. Label instructions of pesticides must be strictly followed, and the product applied only by individuals with currently valid licenses. All applications of pesticides should be made in evening hours, and greenhouse rooms flushed with fresh air before the next morning to minimize exposure to workers. Notice of application should always be posted.
10. Use biological pest control, as an alternative to the application of pesticides, introducing parasitic wasps, predatory insects or mites, nematods, etc. These living animals have limited lives, they cannot be stocked on shop shelves, they have to be ordered and released periodically. Follow carefully the supplier's instructions regarding release and subsequent care of the predators or parasites. The pest must be present, otherwise there is nothing for the predators to feed on or the parasites and nematodes to infest. Predators and parasites should therefore be introduced into a growth area in an early infestation, before a heavy and damaging invasion has developed. These biological control agents cannot give an instant reduction in pest numbers; they need some time to get established. Biological controls are an alternative to pesticides, rather than an additional treatment, they are vulnerable to most insecticides. It relies on predation and parasitism, but typically also involves an active human management role and generally it needs a degree of tolerance to low-level pest populations.

2.1. Maintenance of plants in greenhouses

Greenhouses with satisfactory cooling, heating and supplemental light are suitable for large-scale growth of lines that do not require strict control of environmental conditions, which include most natural accessions (e.g., Col, Ler, Cvi, Ws, Est, Kas, Sha, Kondara, C24) as well as species related to *Arabidopsis thaliana*. However, conditions are often too hot in temperate climates for *Arabidopsis* growth in greenhouses during the summer. Successful plant growth should start with an empty room, cleaned and maintained as follows. Mature or diseased plants, plant debris, used soil, pots and other materials, can shelter pathogen spores or insects from former plantings. After removal of the pest and host materials and the sterilization of the growth area, it is very improbable that any pest or pathogen will survive.

1. Remove and properly discard all plants and other materials in the room.
2. Sweep and hose down the entire room interior (benches, floors, window ledges and windows).
3. Increase the temperature in the room to 40°C for 3-5 days. The temperature setting may be higher, depending on the outside environmental conditions and equipment specification. Lights, fans and cooling pads should be turned off and vents closed during this period.
4. Do not place diseased or older plants in the clean room after the high temperature treatment.
5. Provide supplemental evening and morning light during the winter, since the plants generally require a long photoperiod (at least 12 h) for flowering. In the greenhouse, 16 h photoperiods are typically employed.
6. Use shade cloth during the summer, which helps reduce light intensity and regulate temperature.
7. The recommended growth temperature in the greenhouse is 21-23°C. Night temperatures should be maintained 2-4°C lower than the day temperature.

2.2. Maintenance of plants in growth chambers and growth rooms

Most of the commercial growth chambers precisely control light intensity, photoperiod, temperature (typically $\pm 1^\circ\text{C}$), and often humidity. Custom plant growth rooms provide environmental control similar to that of reach-in chambers. Standard architectural rooms, equipped with supplemental lighting and air conditioning, are popular for reproducing *Arabidopsis* economically. Such rooms must be designed with sufficient light, cooling and ventilation, but typically afford less rigorous control of growth conditions than custom chambers. Such facilities usually allow better control of temperature and light than is offered by a greenhouse, hence, their popularity among *Arabidopsis* researchers. Growth rooms can be maintained within 2-3°C of a set point, while greenhouse temperatures may spike to higher deviations with rapid changes in sunlight, unexpected hot days, etc. As is the case for greenhouses, it is imperative to start a new planting in a growth facility that has been previously emptied and properly cleaned. Hence, the use of chemicals to control pests and loss of plants due to pest infestation is minimized.

1. Remove and discard all plant residues and related materials. Mature or diseased plants, plant debris, used soil, pots and other materials, can shelter pathogen spores or insects from former plantings.
2. Sweep and wipe down the interior with wet paper towel.
3. Make sure the intake and exhaust vents are closed.

4. Apply a sterilizing agent, such as Spor-Klenz[®] Ready To Use, to kill fungal spores if heavy infestation of powdery mildew was present, using a fogger tank (e.g., Tornado[™]/Flex cold fog ULV mist sprayer) through an external access port of the chamber. Read and follow precautionary measures as suggested by the manufacturer of the cold sterilant Spor-Klenz[®].
5. Leave chamber undisturbed overnight, and wipe down the inside of the growth chamber with a wet paper towel the next day.
6. Increase the temperature to 40-45°C for a period of 3-5 days to eradicate/minimize pests.
7. Do not place diseased or older plants in the cleaned chamber.
8. Use continuous light or a long day photoperiod if you wish to accelerate the reproductive cycle. Short days (less than 12 h) favor growth of vegetative tissue and delay flowering.

2.3. Monitoring the environmental growth conditions

The environmental control systems currently offered for greenhouses, growth chambers and growth rooms allow for remote monitoring, control adjustment and alarm notification via internet connections. These features represent a vital tool for avoiding loss of data during plant production and maintaining control of environmental experiments. Installation of remote sensing is recommended for new growth facilities of all types.

In addition to the control and logging systems in place at the growth facilities, environmental growth conditions can be monitored by placing portable data loggers (e.g., the HOBO[®] U14) in growth areas. They can act as a complementary, backup or sole resource for recording environmental data. They can be used to display and record temperature and relative humidity conditions in greenhouses, growth chambers, growth rooms, cold rooms, dry rooms and laboratories. These data loggers offer reliability, accuracy, convenient monitoring and documentation of specific environmental conditions. They can be connected to a computer to quickly display and analyze data.

3. Genetic crosses

Some species of *Arabidopsis*, particularly *A. thaliana*, are mostly self-pollinating, especially in a growth chamber or greenhouse setting where insect populations are minimized. It should be noted that the pollen of *Arabidopsis* does not disperse through the air. Therefore, crossing *Arabidopsis* is mainly conducted through manual emasculation of flowers just prior to flower opening, followed by hand transfer of pollen from the desired male parent to the stigma of the emasculated flower. Although labor-intensive, the manual method remains a reliable technique for achieving cross-pollination.

Species, such as *A. halleri* and *A. lyrata*, have natural self-incompatibility mechanisms, which prevent the plant from self-pollinating and result in obligate outcrossing. For such species, simple maintenance of a genetic stock, cannot easily be accomplished from a single plant, and it is most convenient to start with a small population of founders and perform cross-pollination.

The manual techniques for performing genetic crosses of *A. thaliana* can be generalized to the related species. The use of a magnifying visor (e.g., optical glass binocular magnifier) or dissecting microscope is recommended to visualize floral parts and avoid damage to the pistil.

Genetic crosses can be performed as follows:

1. Select the appropriate parent plants. Choose young plants at early stages of flowering. Avoid using the first flowers in the inflorescence, which are usually less fertile and the smaller flowers produced by mature plants. Crosses may be performed throughout the duration of

the flowering time, however, the crosses will have a higher rate of success during the earlier stages of flowering.

2. Prepare the female parent:
 - a. Select a stem with 2-3 flower buds, in which the tips of the petals are barely visible and before the anthers begin to deposit pollen on the stigma. Using unopened flowers for the female parent is important in order to avoid self-pollination. Shortly after this stage, stamen/pistil length ratio, as well as the timing of anther dehiscence, favor self-pollination and open flowers have most likely been self-pollinated. All flower candidates for female crossing should be examined for presence of released pollen prior to their use in crossing.
 - b. Remove siliques, leaves, and any open flowers above and below the selected buds on the chosen stem with a small pair of scissors; avoid damaging the stem.
 - c. Remove the sepals, petals and all six stamens from the selected flower buds using a precision clamping tweezers, leaving the pistil intact. If the pistil is damaged, it is highly unlikely that the cross will be successful and the flower should not be used.
3. Prepare the male parent: select a newly opened flower with anthers that are dehiscent. These flowers will contain fresh pollen that will contribute to the success of the cross. Remove the flower by squeezing near the pedicel with tweezers.
4. Pollinate the female parent by taking the fully open flower from the male parent and brushing the anthers over the bare stigma of the female parent. Visually confirm that pollen has been deposited on the stigma.
5. Label the crosses, placing a colored tape on the stem of the female plant, noting the male and female parent and the date of the cross.
6. Inspect developing siliques over the next several days. Successful crosses are visible after 3 days when the siliques start elongating. Siliques are ready for harvest once they turn brown, but before they shatter. Siliques should be ready to harvest in about 2-3 weeks after the cross. If siliques are brown, use care, as it is easy to lose all seeds at this stage.
7. Harvest siliques by cutting them with scissors and placing them into a 1.5 mL microcentrifuge tube or a small paper envelope.
8. Air-dry seeds at room temperature, preferably at 20-30% relative humidity, for 1-3 weeks. Thresh seeds if necessary.

4. Seed handling

4.1. Plant isolation, harvesting and preparation for storage

Prevention of cross-contamination among adjacent pots and avoiding the loss of seeds due to shattering are equally important. Plants must be isolated from their neighbors without compromising seed quality. Various methods and devices exist to accomplish these objectives, including Aracons[™], plastic floral sleeves, plastic bags and isolation by space on the open bench. Details of each method are described below.

1. Aracons[™]: Place Aracons[™] over single plants soon after bolting.
2. Plastic transparent floral sleeves (e.g., straight sleeve BOPP 60x40x15 cm for 11 cm diameter pots): Cut four equally spaced holes at the point where the sleeve meets the top of the pot. This will increase aeration and reduce water condensation that may encourage mold growth. Place the sleeve on the pot near the time of bolting, so that all plant inflorescences

are maintained within the sleeve. Plastic sleeves fit snugly around a pot, extend upward, and are wider at the top allowing for expansion of the developing inflorescences. Sleeves made of biaxially-oriented polypropylene (BOPP) are very clear, maintain upright stiffness, and tear easily for harvesting. Fold down the tops of the sleeves about 2 cm to ensure they stay open and stable. If plants grow out above the sleeves and are at high plant density, train the top of the plants back down into the sleeve to avoid contamination. This method is very effective for achieving high densities while maintaining productivity and purity of single lines of different genetic backgrounds.

3. Plastic bags: If plastic bags are used, train inflorescences of non-*erecta* lines into a 4-8 L transparent plastic bag before siliques begin to brown. Bags should be kept open to avoid the accumulation of moisture resulting from transpiration.
4. Open bench growth: Plants can be maintained on the open bench for bulk seed production, keeping all lines separated by adequate space. Avoid disturbance of maturing inflorescences. This method is appropriate when growing natural accessions that are late flowering and develop large and dense canopies (e.g., Sij-1, Monte-1, Amel-1, Anholt-1, Appt-1, Bik-1, Bl-1, Do-0).

The simplest procedure is to wait until the entire inflorescence has browned before harvesting. However, some siliques may shatter naturally and seed will be lost. Harvest seeds only after the soil in pots or flats has been allowed to dry. It should be noted that delays in harvesting following physiological maturation of the plant result in seed deterioration, especially under non-optimal environmental conditions. Seeds from individual siliques can be harvested after the fruits have turned completely yellow, if rapid turnover is required. However, such seeds have high levels of germination inhibitors. Since formation and maturation of siliques occur over time, early siliques can be harvested before later ones mature. Harvest for each of the four isolation methods is as follows:

1. Aracons[™]: Slide the plastic cylinder off, and then cut off the dry inflorescence above the cone device in a threshing sieve.
2. Plastic floral sleeves: While holding the pot, cut away and discard plastic sleeve. Cut the dry inflorescences and place them in a threshing sieve.
3. Plastic bags: Cut the entire plant off at its base. Shake the seeds into the bag; inflorescences can be gently hand-pressed from the outside, and the seeds will fall to the bottom of the bag. Most of the dry inflorescences can be removed from the bag by hand before seeds are sieved to separate them from chaff.
4. Open-bench: Cut off the entire inflorescence at its base, and carefully place into a 4-8 L or larger transparent plastic bag, depending on the size of the bulk of plants.

The major factors influencing seed longevity are: 1) genotype, 2) environmental conditions during seed maturation, harvesting and seed handling and 3) seed storage conditions. Harvested seeds should be processed promptly (including threshing, cleaning, drying and packaging) and placed into storage.

Seeds should be threshed when the seed moisture content is approximately 10%, to minimize seed damage during threshing. This seed moisture content will be reached when all plant material appears to be dry. Hand, rather than machine threshing, is recommended mainly because threshing machines need rigorous cleaning between lines to avoid sample cross-

contamination, require very careful adjustment and do not accommodate the variable size of *Arabidopsis* seeds well. The hand method is performed as follows:

1. Set a large, clean, white paper on a bench or table for collection of the threshed seeds.
2. Place a clean threshing hand sieve (e.g., U.S. Standard Stainless Steel Test Sieve No 40) on top of the paper.
3. Place dry plants directly onto the sieve. If plants are larger than the sieve, they can be cut into pieces that fit the screen.
4. Crush plants using hands to remove all the seeds from siliques. Discard plant material.
5. Sieve seeds through the mesh repeatedly until they are clean and free of chaff. After sieving, the seeds are still likely to be mixed with soil and plant residue. A combination of additional sieving, gentle blowing and visual inspection can be employed to clean the seeds completely.
6. Clean small samples by hand with the aid of a pointed tool on an opaque glass plate illuminated from below, if needed.
7. Place cleaned seed samples in small labeled manila envelopes (e.g., 6 cm x 9 cm) or small open glass jars (125 mL) or other containers to allow seeds to air dry. Do not use plastic due to static effects.

The ideal moisture content of seeds for storage is 5-6%. Higher moisture content can cause seed deterioration. There are many methods available for drying seeds. The recommended method is to air-dry the seeds at room temperature and approximately 20% relative humidity for 1-3 weeks. The moisture content of *Arabidopsis* seeds stored in open containers corresponds to the room humidity. *Arabidopsis* seeds behave in a similar way to crop seeds with similar chemical composition. Low relative humidity (20-30%) is necessary for seeds to reach the desired moisture content. Seed moisture content can be determined by several methods and it is calculated as the loss in weight as a percentage of the original weight of seeds. Seed packaging for storage can be accomplished as follows:

1. Use polypropylene cryovials with threaded lids and gaskets (e.g., 2 mL screw cap micro tubes) for convenient, safe and permanent seed storage. They hold large numbers of seeds, seal tightly, are moisture-proof and can be resealed many times.
2. Label each vial with pertinent information including date of storage, use printed labels or permanent marker.
3. Determine stored seed quantities (approximately $50 \mu\text{l} = 25 \text{ mg} = 1,250$ seeds).

4.2. Seed storage and preservation

The general conditions for preserving optimal viability of seeds have been well defined. Seed storage principles for *Arabidopsis* are similar to those for other plants, with the caveat that the small seeds rehydrate very rapidly if exposed to high humidity. When seeds deteriorate, they lose vigor and eventually the ability to germinate. The rate of this 'aging' is determined by interactions of the temperature and moisture content at which seeds are stored, and unknown cellular factors that affect the propensity for damage reactions.

Rapid deterioration of seeds has not been observed for the diverse collections currently maintained at ABRC. However, experience regarding the effect of genotype is limited. A large number of genes involved in embryogenesis, reserve accumulation and seed maturation have been identified. Conspicuously, seeds of the abscisic acid-insensitive mutants fail to degrade

chlorophyll during maturation and show no dormancy, leading to low desiccation tolerance and poor longevity. *Arabidopsis* seeds should retain high viability for long storage periods, under proper conditions. With the increase of storage temperature and seed moisture content, the lifespan of the seeds decreases. Seeds left at room temperature and ambient relative humidity lose viability within approximately two years. Seed stored dry at 4°C or -20°C should last decades. Below are three storage options for safe seed preservation.

1. For active collections which are accessed often, store seeds at 4°C and 20-30% relative humidity. Control of humidity is typically achieved by a dehumidification system in the cold room. Note that the control of relative humidity provides a safety factor in case seed containers are not sealed properly.
2. For long-term or archival storage, the recommended temperature is sub-zero, preferably -20°C, and also preferably 20% relative humidity.
3. For open containers such as envelopes, seeds can be stored at 15-16°C, with a relative humidity maintained very carefully at 15%. Under this controlled environment, seeds will maintain suitable low moisture content. Storing seeds at relative humidity < 15% will not increase shelf-life and may actually accelerate deterioration.

When vials are removed from cold storage, condensation of moisture on the seeds and subsequent damage may occur. For vials stored at 4°C, sealed vials must always be warmed to room temperature before opening. For vials stored at -20°C rapid re-warming (placing the sealed vial in a 37°C water bath for 10 min) is a recognized method to minimize frost damage. If possible, working with seed stocks should take place at low (20-30%) relative humidity. If accumulation of condensation is suspected, vials should be left open in the dry room until seeds have equilibrated before returning the vials to cold storage.

4.3. Seed quality control

The purity and physical integrity of seeds and the presence of pests and seed-borne diseases (especially some fungal diseases) can be detected by visual examination with the naked eye, magnifying lenses or using a dissecting microscope. For a rigorous assessment, spread the seeds on white paper under a well-lit microscope. Generally, gray or white coloration on the seed surface indicates fungal contamination. Discard seeds if possible; otherwise sterilize seeds with fungicides before planting. Do not discard shriveled, small, irregular shaped and other colored seeds that might correspond to specific mutations, assuming that the seeds were produced under optimal conditions.

Seed viability should be monitored at regular intervals by conducting germination tests under a standard set of conditions. It is recommended that seeds in long-term storage under the optimal preservation standards should be monitored at least every ten years. Seeds in short-term storage should be monitored at least every five years.

A germination test for *Arabidopsis* can be conducted in 3-7 days to determine the proportion of seeds in a sample that will produce normal seedlings. Tests should be carried out before seeds are stored, so that poor quality samples can be recognized. *Arabidopsis* seeds may fail to germinate because they are dormant or because they are defective or non-viable. Dormant seeds can be distinguished because they remain firm and in good condition, while non-viable seeds soften and are attacked by fungi. Most widely used lines have moderate dormancy, and cold treatment, also called stratification, may not be required for germination when planting older

seeds of these lines. However, a cold treatment at 4°C for 3 days will improve the rate and synchrony of germination. The use of an extended cold treatment of approximately 7 days is especially important for freshly harvested seeds, which have more pronounced dormancy. An extended cold treatment is also necessary for certain natural accessions. Cold treatment of dry seeds is usually not effective in breaking dormancy.

Initial germination rate should exceed 80%, but may be lower for some lines. Mutations in a significant number of genes, mostly involved in biosynthesis and signaling pathways of certain hormones, affect seed germination and/or dormancy. A germination test can be performed as follows:

1. Label the bottom of a 10 cm diameter plastic Petri plate with name and date.
2. Place two layers of absorbent paper (e.g., filter paper 10 cm diameter) in the bottom of the plate and moisten with distilled water. Remove excess water.
3. Distribute 100 seeds evenly on the surface of the paper. Seal the plate with Parafilm or clear tape, to prevent drying.
4. Stratify seeds by placing the plates at 4°C for 3 days.
5. Move the plates to an illuminated shelf or to a growth chamber under standard light and temperature conditions. Environmental conditions for seed germination tests are the same as for growing plants. Two replicates of 100 seeds each provide reliable germination estimates. Cases in which observed germination is < 80% may warrant follow-up testing.
6. Record germination percentage after 3 to 7 days by dividing the number of seedlings by the total number of seeds, and multiplying by 100.

Germination tests can also be performed on solid media, such as MS, described in subheading 1.1.

These methods are used by the ABRC for handling plants and seeds. If you have any questions concerning the above procedures, feel free to contact us at abrc@arabidopsis.org

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