Video Article

Standardized Method for High-throughput Sterilization of Arabidopsis Seeds

Benson E. Lindsey III*[1], Luz Rivero*[1], Chistopher S. Calhoun[1], Erich Grotewold[1,2], Jelena Brkljacic[1]

[1]Arabidopsis Biological Resource Center, Center for Applied Plant Sciences, The Ohio State University

[2]Department of Molecular Genetics, Center for Applied Plant Sciences, The Ohio State University

*These authors contributed equally

Correspondence to: Jelena Brkljacic at brkljacic.1@osu.edu

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Abstract

Arabidopsis thaliana (Arabidopsis) seedlings often need to be grown on sterile media. This requires prior seed sterilization to prevent the growth of microbial contaminants present on the seed surface. Currently, Arabidopsis seeds are sterilized using two distinct sterilization techniques in conditions that differ slightly between labs and have not been standardized, often resulting in only partially effective sterilization or in excessive seed mortality. Most of these methods are also not easily scalable to a large number of seed lines of diverse genotypes. As technologies for high-throughput analysis of Arabidopsis continue to proliferate, standardized techniques for sterilizing large numbers of seeds of different genotypes are becoming essential for conducting these types of experiments. The response of a number of Arabidopsis lines to two different sterilization techniques was evaluated based on seed germination rate and the level of seed contamination with microbes and other pathogens. The treatments included different concentrations of sterilizing agents and times of exposure, combined to determine optimal conditions for Arabidopsis seed sterilization. Optimized protocols have been developed for two different sterilization methods: bleach (liquid-phase) and chlorine (Cl_2) gas (vapor-phase), both resulting in high seed germination rates and minimal microbial contamination. The utility of these protocols was illustrated through the testing of both wild type and mutant seeds with a range of germination potentials. Our results show that seeds can be effectively sterilized using either method without excessive seed mortality, although detrimental effects of sterilization were observed for seeds with lower than optimal germination potential. In addition, an equation was developed to enable researchers to apply the standardized chlorine gas sterilization conditions to airtight containers of different sizes. The protocols described here allow easy, efficient, and inexpensive seed sterilization for a large number of Arabidopsis lines.

Video Link

The video component of this article can be found at https://www.jove.com/video/56587/

Introduction

Arabidopsis thaliana (Arabidopsis) is a prime model organism for basic and applied research in plant biology[1,2,3]. While standard conditions for Arabidopsis growth have been well established[4], the effect of seed sterilization on seed viability has not been rigorously tested. Solid media in plates or boxes is routinely used to facilitate growth of Arabidopsis seedlings for many experimental applications, such as identification of homozygous lethal mutants in a segregating population, observation of shoot and root phenotypes at early stages, isolation of pathogen-free tissue, collection of large amounts of seedling tissue, selection of transformants or drug-resistant plants and evaluation of germination[1,2,3,4]. Seeds harvested from plants grown in a greenhouse or growth chamber are occasionally contaminated with microorganisms and dust. Growth of Arabidopsis seedlings on different types of sterile media requires prior seed sterilization to remove microbial contaminants such as fungi and bacteria present on the seed surface. The use of an effective seed sterilization regime is critical for a balance of high germination, minimum contamination, and vigorous plant growth.

The two most common methods used for Arabidopsis seed sterilization are based on commercial bleach (liquid-phase) and chlorine gas (vapor-phase). Various procedures have been employed for both liquid-phase sterilization[1,4,5,6,7,8,9,10] and vapor-phase sterilization of Arabidopsis seeds[6,10,11,12,13,14,15,16]. However, while these procedures have been effective in accomplishing seed sterilization of the utilized genotypes, a detailed analysis of the effect of different sterilization treatments on the seeds of different genotypes has not been reported. Therefore, the optimization of these sterilization procedures is required to define conditions in which efficient sterilization is combined with high germination rate.

The Arabidopsis Biological Resource Center (ABRC) is uniquely positioned to a) test seed viability of a variety of different genotypes in the collection and b) take advantage of the quality control procedures applied internally and in response to user feedback about seed germination. The objectives of the experiments presented here were to determine the effects of various sterilization methods on seed germination of a range of Arabidopsis genotypes. Optimized sterilization procedures which result in high seed germination rates while maintaining minimal pathogen contamination are presented for both bleach and chlorine gas sterilization.

Protocol

1. Preparation of 1x Murashige and Skoog (MS) medium

1. Add 4.31 g of MS basal salt mixture, 10 g sucrose 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. Check and adjust pH to 5.7 using 1 M potassium hydroxide (KOH). Add distilled water to make 1 L.
2. Divide the media into two 1 L bottles, 500 mL each. Add 5 g of agar to each bottle. Keep the lid loose.
3. Autoclave for 20 min at 121 °C, 15 psi with a magnetic stir bar in the bottle.
4. After autoclaving, place the bottles on a stir plate at low speed, and allow the MS medium to cool to 45-50 °C (until the bottle can be held with bare hands).
5. Starting from this step, perform all the steps in sterile conditions in a laminar flow hood. Add 500 µL Gamborg's Vitamin Solution to each bottle and stir the MS medium to evenly distribute the vitamin solution.
6. Pour enough media into plates to cover approximately half of the depth of the plate.
7. Allow the plates to cool at room temperature for about 1 h to allow the agar to solidify.
   Note: 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 an airtight container.

2. Sterilization of Arabidopsis Seeds with Bleach

1. Prepare the MS plates in accordance with section 1 of the protocol. Autoclave 100 mL of distilled water at the same time as the MS Media. Use this later on as rinse water and to help suspend the seeds to aid in plating.
   Note: If desired, 0.8% agar blend (w/v) (*e.g.* phytagar) can also be autoclaved in this step. The agar blend can be substituted for distilled water during plating (Step 2.5.3.). The extra viscosity of the agar blend makes it easier to space seeds on the plate or plant in rows if necessary.
2. Prepare 50% (v/v) bleach solution to be used for sterilizing the seeds. To dilute bleach, add 100 mL of bleach to 100 mL of distilled water. Add 50 µL of Tween 20 detergent to the bleach solution.
   Note: Prepared bleach solution can be stored for up to a month as long as it is only opened in sterile conditions.
3. Aliquot 100 seeds into a 1.5 mL microcentrifuge tube.
4. **Sterilize the seeds using a 50% bleach solution.**
   1. In a laminar flow hood, add 500 µL of the 50% bleach solution to the microcentrifuge tube containing the seeds. Tap the bottom of the tube to suspend the seeds in the bleach solution.
      Note: Alternatively, a rotator or platform shaker may be used to keep the seeds suspended.
5. **Rinse the bleach solution from the tube.**
   1. After 10 min, remove the bleach solution from the microcentrifuge tube using a pipette or an aspirator fitted with a pipette tip on the end.
   2. Add 500 µL of sterile distilled water to the tube. Close the tube and invert to mix. Allow seeds to settle to the bottom of the tube. Once seeds have settled to the bottom of the tube carefully remove the bleach solution by pipetting. Repeat this rinsing process 6 times.
   3. Add 1 mL of autoclaved distilled water to the tube to suspend the seeds.
6. **Plate the sterilized seeds on MS plates.**
   1. In a laminar flow hood, label the bottom of the MS plate with the stock name and the current date.
   2. Pour the seeds from the microcentrifuge tube onto the MS plate. Spread the seeds around the MS plate using a sterile, single-use inoculating loop or a sterile pipette tip.
      Note: If seeds are to be sown in rows, a pipette with a 200 µL tip can be used to individually place seeds in the desired positions. To improve the flow of seeds, the end of the pipette tip can be trimmed by 3-5 mm using scissors. Any misplaced seeds or clusters of seeds can then be repositioned or separated using a sterile single-use inoculating loop.
   3. Place the MS plate at the back of the of the laminar flow hood with the lid half closed. Allow the excess water to evaporate from the MS plate.
   4. Place the lid on the MS plate. Seal the MS plate by wrapping the plate with microporous paper surgical tape (see the **Table of Materials**).

3. Sterilization of Arabidopsis Seed with Chlorine Gas

1. Prepare the MS plates in accordance with section 1 of the protocol. Autoclave 100 mL of distilled water at the same time as the MS Media; this will be used later to help suspend the seeds to aid in plating.
   Note: If desired, 0.8% agar blend (w/v) (*e.g.* phytagar) can also be autoclaved in this step. The agar blend can be substituted for distilled water during plating (Step 3.6.2.). The extra viscosity of the agar blend makes it easier to space seeds on the plate or plant in rows if necessary.
2. **Before starting sterilization, calculate the amounts of bleach and hydrochloric acid (HCl) required to produce chlorine gas.**
   1. Calculate the amount of HCl needed to produce the 6.1% Cl_2 required for sterilization.
   2. Use the following formula:

      $$V_{HCl} = \left(\frac{\%Cl_2 x\ V_{container}}{149.4\ x\ (100 - \%Cl_2)}\right) mL$$

      with 7,000 mL as the volume of the sterilization container and 6.1 as $\%Cl_2$; the volume of HCl is calculated to be 3 mL.

Note: A spreadsheet programmed to carry out the calculation for different volumes of the container and $\%Cl_2$ is provided as **Supplemental Table 1**.

3. Aliquot 100 seeds into 0.5 mL microcentrifuge tube. Close the caps for each vial, place the vials in a plastic rack and set aside.
Note: Seeds can be stored for an extended period of time at this point as long as they are stored in proper conditions. Storage conditions can be found in Section 3.3.2 of the protocol by Rivero and colleagues[4]. 96-well plate format can also be used.
4. **Prepare the materials necessary to perform chlorine gas sterilization.**
    1. Obtain bleach and HCl from their storage locations.
    2. Cut a strip of large paraffin film (see the **Table of Materials**) to use in step 3.5.3. to seal the sterilization container.
    3. Place the plastic container with lid in which the sterilization will take place inside a fume hood. Open the caps on all the seed vials and place the entire seed rack inside the plastic container.

5. **Perform chlorine gas sterilization at room temperature.**
Caution: Work with bleach and acid separately. Do not leave either bottle uncapped to reduce risk of spills. Use appropriate personal protective equipment (PPE) including gloves and a lab coat. If either bleach or acid splashes on gloves, change gloves before handling other materials. Always remove gloves in the fume hood in case of bleach or acid contamination.
    1. Place a 250 mL beaker inside the container and add 100 mL of bleach.
    Note: The reaction between HCl and bleach requires at least 22 volumes excess of bleach. The reaction will consume the HCl and bleach by releasing Cl_2 gas with sodium chloride (NaCl) and water as byproducts. Using a large excess of bleach allows the consumption of additional HCl during the venting period, which decreases the amount of sodium bicarbonate ($NaHCO_3$) needed to neutralize the solution for disposal.
    Caution: The beaker should be at least twice the total liquid volume of bleach + HCl. This prevents splashes escaping the beaker during the next step, which can damage seeds, bleach clothes, or burn exposed skin.
    2. Add 3 mL of HCl to the beaker containing the bleach.
    Caution: The initial reaction will produce bubbles, especially at gas concentrations higher than 6.1%. A long-sleeved lab coat is necessary for this step.
    3. Close the sterilization container and seal it with paraffin film immediately.
    4. Monitor the sterilization container during the sterilization time to ensure gas accumulation; the accumulation of chlorine gas should be visible as a faint yellow haze inside the container.
    Caution: Periodically check the sterilization container to ensure that pressure inside has not unseated the lid or paraffin film. If the lid has come unseated or the paraffin film has come loose, close the lid and carefully wrap the container with an additional layer of paraffin film.
    5. After the 1 h-sterilization period, open the container by removing the paraffin film and opening the lid on one corner. Allow the container to vent for 3 h to complete the reaction and eliminate the chlorine gas.
    6. Close the caps of the all the microcentrifuge tubes in the seed rack.
    Note: Sterilized seeds can be stored until time of plating as long as they are stored in dry condition.
    7. Remove the seed rack and place it in a laminar flow hood.
    8. Neutralize chlorine gas reaction
        1. Add 1.5 g $NaHCO_3$ powder slowly to the beaker containing the bleach/HCl solution and stir with a glass rod to dissolve the $NaHCO_3$ into solution. Continue adding $NaHCO_3$ until carbon dioxide (CO_2) gas bubbles have stopped forming.
        Caution: Add slowly to prevent splashes. Use appropriate PPE including gloves and a lab coat.
        2. Test the pH of the solution using pH strips or a pH meter. Add additional $NaHCO_3$ if necessary until the pH of solution is neutral (pH 7.0). At this point the solution can be removed from the fume hood and disposed of according to all applicable disposal guidelines.
        Caution: If ANY smell is noticed during disposal, then the solution should immediately be returned to the fume hood.

6. **Plate the sterilized seeds on MS plates.**
    1. In a laminar flow hood, label the bottom of the MS plate with the stock name and the current date.
    2. Add 500 µL of sterilized distilled water to each microcentrifuge tube to suspend the seeds. Pour seeds on to the MS plate and spread the seeds evenly around the plate using a sterile, single-use inoculating loop or a sterile pipette tip.
    3. Place the MS plate at the back of the of the laminar flow hood with the lid half closed. Allow the excess water to evaporate from the MS plate.
    4. Place the lid on the MS plate. Seal the MS plate by wrapping the plate with microporous paper surgical tape.

4. Growth of Arabidopsis on MS plates

1. Place the plates with the lid on top for three days at 4 ˚C and ambient humidity.
Note: This process is called stratification and serves to synchronize the germination of individual seeds.
2. **Transfer the plates to the growth environment.**
    1. Maintain temperature at 23 °C and light intensity at 120 - 150 µmol/m^2s with 16 h light/ 8 h dark photoperiod. Place plates flat into the grow environment with the lid on the top so the roots grow into the medium.

3. Let the seedlings on plates grow for 8 days.
Note: An 8 day growing period allows late germinating seeds to germinate. Plates can be scored sooner than 8 days if all seeds have germinated.
4. **Score the germination rates.**
    1. Record the number of seeds that have germinated and that have not germinated. Calculate germination rate by dividing the number of seeds that germinated by the total number of seeds on the plate.

Note: Germination is counted when the radicle has projected outside the seed coat and the two cotyledons are visible.

2. Also count the number of seeds affected by mold to determine the effectiveness of the sterilization conditions.

Representative Results

Arabidopsis seeds collected from an open field, greenhouse or growth chamber are sometimes contaminated by various microorganisms like fungi and bacteria[1,4]. Thus, germinating seeds on sterile media can be particularly challenging due to contamination of the plates, especially when the seed supply is limited. The optimized protocol for both bleach and chlorine gas sterilization, the results of which are presented below, minimizes this problem and preserves the viability of seeds required for high-throughput applications.

Effects of bleach sterilization on germination of Arabidopsis Col-0 seeds

Bleach is the most commonly used agent for seed sterilization in numerous plant species. The optimal concentration of the sterilization agent and the exposure time varies between species. A number of protocols have been employed using bleach for sterilization of Arabidopsis seeds[1,4,5,6,7,8,9,10]. Four different concentrations of bleach with five different exposure time periods were tested and the results are presented in **Figure 1**. The treatments were applied to Columbia wild-type (Col-0) seeds. The effect of bleach concentration on germination of Col-0 seeds varied depending on the time of sterilization as demonstrated by a significant interaction between bleach concentration and time of sterilization (**Figure 1**, $P < 0.001$, ANOVA -Analysis of variance).

In the experiments with sterilization times between 5 and 10 min, treatments with all bleach concentrations resulted in equally high germination rate of Col-0 seeds (**Figure 1**). High germination rates were also observed for household bleach concentrations of 40% and 50% for all sterilization times. Treatments with 80% and 100% bleach for periods longer than 10 min resulted in a significant decrease in germination rate compared to the shorter soaking times ($P < 0.01$, ANOVA). Furthermore, for both 80% and 100% bleach treatments for 20 min, germination was significantly decreased compared to the corresponding 40% and 50% bleach treatments ($P < 0.001$, ANOVA).

Seeds displayed various levels of bleaching and shriveling when using high bleach concentrations for 15 min or longer. In addition to relatively high (up to 32%) seed mortality, germinated seeds sterilized in these conditions often showed growth defects, reflected in a failure of the cotyledons and hypocotyls to unfold and elongate, resulting in developmental arrest. Most treatments (14 out of 20) were completely mold-free, resulting in overall mold level average of 0.21% ± 0.003 (**Table 1**).

A treatment with 50% bleach and a soaking time of 10 min was selected as the best sterilization regime because it combined high germination percentage with good inhibition of surface pathogen growth. This treatment was selected to test the effect of bleach sterilization on different mutant lines as described below.

Effects of chlorine gas sterilization on germination of Col-0 seeds

To optimize chlorine gas sterilization conditions, three different concentrations of chlorine gas were used to sterilize Col-0 seeds for two periods of time (**Table 2**). The gas concentration was calculated based on the volume of concentrated HCl and the volume of the sterilization container using the following equation:

$$\%Cl_2 = \left(\frac{149.4 \; x \; V_{HCl}}{(149.4 \; x \; V_{HCl}) + V_{container}}\right) x \; 100$$

This equation was derived using the ideal gas law assuming 12.3 M HCl, a temperature of 23 °C and standard atmospheric pressure of 101.3 kPa.

The effect of chlorine gas concentration on germination of Col-0 seeds was shown to depend on the time of sterilization as indicated by a significant interaction between the time and the concentration factors (**Figure 2A**, $P < 0.01$, ANOVA). Chlorine gas concentration had no significant effect on germination of Col-0 seeds subjected to 1 h sterilization. With this sterilization time, all concentrations of chlorine gas promoted similarly high levels of germination, above 85% ($P > 0.05$, ANOVA). On the other hand, a 3 h-long sterilization resulted in a significant decrease in the germination rate for the highest concentration of chlorine gas, compared with the two lower gas concentrations ($P < 0.05$, ANOVA). These results indicate that treatments of Arabidopsis seeds with any of the tested chlorine gas concentrations for 1 h, or with gas concentrations below 16.5% for 3 h, are equally effective in preserving seed viability since germination rates were always greater than 82%. However, sterilizing seeds for 3 h with 16.5% gas was detrimental to seed germination.

The incidence of mold was also dependent on gas concentration and exposure time. Mold growth was effectively inhibited with the relatively high concentrations of 6.1% and 16.5% chlorine gas for 1 h-long treatment and with all gas concentrations for 3 h (**Figure 2B**).

Based on these results, a treatment with a gas concentration of 6.1% for 1 h (**Table 2**) was selected as the best vapor-phase sterilization condition to test the effect of gas sterilization on different mutant lines, since it combined high germination rate (85%) with very low level of mold contamination (0.02%).

Effects of bleach and chlorine gas sterilization on seeds with different germination potential

Statistical analysis showed that the germination response to sterilization methods was dependent on the germination potential of the lines (**Figure 3A**, $P < 0.01$, ANOVA). Neither bleach nor chlorine gas sterilization reduced germination rate of seeds with high germination potential (Groups 4 and 5). Neither treatment had an effect on the already low germination rate of the group with the lowest germination potential (Group 1). In contrast, chlorine gas sterilization resulted in a significant reduction by approximately 12-18% ($P < 0.01$, ANOVA) in the germination of seeds with intermediate germination potential (Groups 2 and 3). Bleach sterilization also decreased germination rate by 13% of Group 2, but it did not decrease the germination rate of Group 3. Although there was no significant difference in germination rate between the bleach and chlorine gas treatments in any germination group (**Figure 3A**, $P > 0.442$, ANOVA), seeds sterilized using bleach did have a slightly higher germination rate than gas-sterilized seeds in all germination groups.

Sterilization treatments significantly altered ($P < 0.001$, ANOVA) the percent of seeds affected by mold (**Figure 3B**). Both chlorine gas and bleach sterilization resulted in less mold growth ($P < 0.05$, ANOVA) compared to no sterilization. There was no difference in mold level detected between gas and bleach sterilization in any group (**Figure 3B**, $P > 0.4$, ANOVA).

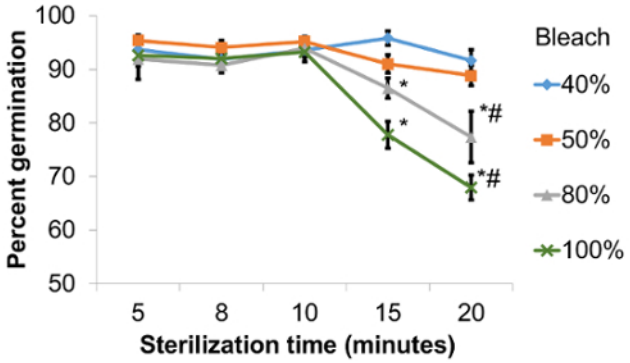

Figure 1: Effects of bleach concentration and sterilization time on germination of Arabidopsis Col-0 seeds. Values are means ± SD obtained from 5 independent replications of the experiment. *indicates significant differences relative to the 5 min soaking duration for a range of bleach concentrations ($P < 0.01$, ANOVA). #indicates significant differences of 80% and 100% bleach concentrations relative to 40% and 50% for a 20 min time period ($P < 0.01$, ANOVA). Please click here to view a larger version of this figure.

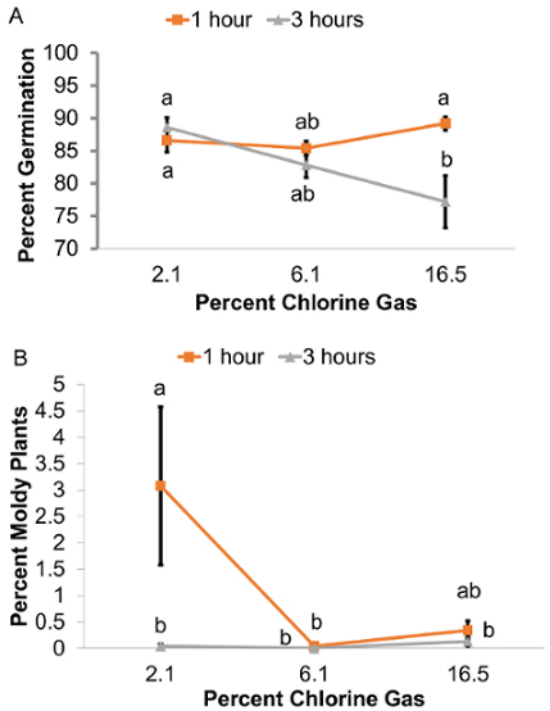

Figure 2: Effects of chlorine gas concentration and sterilization time on Arabidopsis Col-0 seeds. (**A**) Germination rate and (**B**) Mold level. Error bars represent means ± SD obtained from 5 biological and 5 technical replications of the experiment. Means that do not share a letter are significantly different ($P < 0.05$, ANOVA). Please click here to view a larger version of this figure.

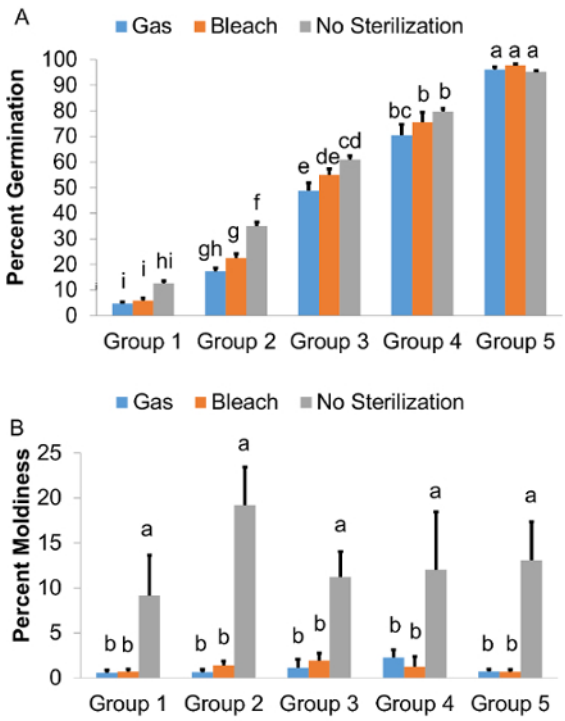

Figure 3: Effects of bleach and chlorine gas sterilization on seeds with different germination potential. (**A**) Germination rate and (**B**) Mold level. The 100 SALK T-DNA lines were classified into five groups according to their germination potential defined as the germination rate in the absence of any sterilization agent. The groups based on the germination rate were as follows: Group 1 (0-20%), Group 2 (21-50%), Group 3 (51-70%), Group 4 (71-90%) and Group 5 (91-100%). The lines were chosen randomly and their germination potential did not depend on genotype. Values are means ± SD obtained from three independent replications of the experiment. Letters ("a", "ab", "c", etc.) above each value indicate the statistical groupings of category means. Means that do not share a letter are significantly different ($P < 0.05$, ANOVA). Please click here to view a larger version of this figure.

Sterilization Time (min)	Bleach Concentration (%)			
	40	**50**	**80**	**100**
5	0.00%	0.00%	0.00%	2.13%
8	0.00%	0.00%	0.00%	0.00%
10	0.00%	0.50%	0.00%	0.36%
15	0.00%	0.00%	0.00%	0.68%
20	0.19%	0.28%	0.00%	0.00%

Table 1: Mold level of bleach-sterilized Col-0 seeds.

Bleach (mL)	HCl (mL)	Time (h)	% Chlorine gas (mol Cl_2/mol total gas)
25	1	1	2.1
25	1	3	2.1
100	3	1	6.1
100	3	3	6.1
200	9	1	16.5
200	9	3	16.5

Table 2: Chlorine (Cl_2) gas sterilization treatments on Columbia wild-type seeds using a 7 L container.

Supplemental Table 1: A spreadsheet programmed to carry out the calculation for different volumes of the container and %Cl_2. Please click here to download this file.

Discussion

When growing Arabidopsis seeds on sterile media, some form of sterilization must be applied. Both bleach and chlorine gas sterilization treatments result in similar germination rate and mold growth inhibition. Neither sterilization method causes a significant reduction in germination rate for seeds with high germination potential; however, bleach sterilization is recommended for lines with lower germination potential (20-70%), due to the small, albeit non-significant, improvement in germination rate compared to gas sterilization (**Figure 3A**).

Sterilizing Arabidopsis seeds with bleach concentrations from 40-100% for up to 10 min provides satisfactory germination percentages and effective mold suppression. Although bleach concentrations less than 40% provide adequate sterilization for most seed lots, using a concentration of 40% or higher guarantees effective sterilization of even heavily contaminated seed lots. It is important not to exceed 10 min of sterilization when using bleach concentrations equal or higher than 80% to avoid high seed mortality and defects in seedling development.

Treating Arabidopsis seeds with chlorine gas concentrations of 6.1% or 16.5% for 1 h results in high germination rates and adequate mold elimination. Low chlorine gas concentration (2.1%) can be used successfully by increasing duration of sterilization to 3 h.

When few lines need to be sterilized, liquid sterilization in a solution of 50% bleach for 10 min is recommended. For greater numbers of lines, gas sterilization with a gas concentration of 6.1% for 1 h is a better option since many lines can be sterilized quickly and easily with less manipulation.

Our results provide standardized conditions for sterilizing both large number of seeds of different genotypes and the seeds with lower germination potential. The only limitation of these sterilization techniques is that they cannot be applied to the seeds with germination rates less than 20% due to extensive seed mortality. Alternative methods, such as sonication[17], to increase germination rate in the absence of sterilization might be beneficial in those cases.

Disclosures

The authors have nothing to disclose.

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