***Arabidopsis thaliana* for Spaceflight Applications—Preparing Dormant Biology for Passive Stowage and On-Orbit Activation**

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ABSTRACT

Biological experiments on-orbit that demonstrate the effects of gravity on plants require precise control of the initiation of plant development. Preserving seed dormancy is critical to experiments that endeavor to study the effects of the orbital environment, independent of contributions from either a normal gravity, or launch. However, spaceflight experiments are often tightly constrained with respect to the configuration of the biology and associated hardware, and it is rarely possible to launch dry seeds separated from their growth substrate. Described here are techniques established to maintain viable seeds that can remain dormant for up to a month at room temperature, and hydrated on the surface of solid, Phytigel growth medium. The configuration can also accommodate a brief

(less than one minute) exposure to light during the quiescent period for quick inspection for any breaks in dormancy, and for contamination. The data presented outline the preparation of sealed, Phytigel media plates of dormant *Arabidopsis thaliana* seed that can be activated *in situ* when unwrapped and installed within a lighted growth habitat. These protocols were developed primarily for spaceflight scenarios where seeded plates must be prepared ahead of time and kept at ambient temperatures. However, these protocols can be adapted for any field application where it is desirable to transport dormant, seeded plates to a remote location where it would not be possible to prepare sterile culture plates.

INTRODUCTION

Understanding plant growth and development on-orbit is central to humankind's space exploration agenda. The effects of microgravity on plants have been explored for several decades (Paul et al., 2013a; Ričkienė, 2012; Wheeler, 2011; Wolverton and Kiss, 2011; Wyatt and Kiss, 2013); however, to fully understand the effects of microgravity, it is ideal to observe differences in plants that have been developed entirely in the microgravity environment without prior exposure to Earth's 1 g force. This endeavor entails launching dormant seeds, germinating on-orbit, allowing them to grow in microgravity conditions,

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and comparing them to plants grown exclusively on Earth as a ground control.

One challenge in conducting such experiments in space is ensuring that the configuration is compact, lightweight, safe, and uses minimal crew time. Solid nutrient media (such as with agar or Phytigel) contained in Petri plates has been used in plant research for decades in terrestrial (Barrett-Lennard and Dracup, 1988; Lodha and Netravali, 2005) and orbital research (Paul and Ferl, 2002; Stout *et al.*, 2001; Zupanska *et al.*, 2013) by providing the necessary components required for healthy plant growth. Most importantly, this configuration is lightweight, completely contained, and allows easy access to all plant tissues – especially the roots.

Another consideration is the ease of operation for both the investigators and the astronauts on-board. Mounting an experiment to the International Space Station (ISS) can be complicated given the many conditions required for a successful launch. Hence, for the investigator, it is essential that the biological elements can withstand unpredicted changes in the launch schedule before actual lift-off and delays in operations on-orbit, as such events are common due to a myriad of technical requirements. Therefore, the ideal system should allow for a few days of flexibility without completely voiding the experiment. For example, if the investigator is interested in studying three-day-old plants, and germination is scheduled to be initiated on the ground prior to space vehicle launch, there is always a risk that upon reaching the critical time frame the plant may still be on voyage to the ISS and not accessible for any in-flight operations. Thus, the capability to send dormant seeds that can be initiated for growth at a convenient and flexible time is needed in traversing the challenging environment of spaceflight or other remote environments. An additional advantage of designing the experiment configuration with this flexibility is that the process promotes ease of operations by crew members on-board the ISS.

We have used Phytigel media plates made with commercially available 100 mm square Petri dishes and dormant *Arabidopsis thaliana* (arabidopsis) seed in several spaceflight and remote expedition applications with great success (Abboud *et al.*, 2013; Bamsey *et al.*, 2009; Paul *et al.*, 2013b; Paul *et al.* 2012). The seed dormancy

protocol was primarily developed for the Advanced Biological Research System/GFP imaging system (ABRS/GIS) on-board the ISS, and the comparable ground control unit installed in the Orbital Environmental Simulator (OES) at Kennedy Space Center (KSC) for the arabidopsis spaceflight experiment. However, the results of our prolific assays may be useful for other applications – not only for spaceflight scenarios with different growth payload hardware, but also whenever dormant plant biology may prove favorable. A non-spaceflight example is the deployment of plated arabidopsis to Devon Island in the Canadian high arctic with the Houghton Mars Project (e.g., Bamsey *et al.*, 2009).

MATERIALS AND METHODS

Preparation of Seeds and Plates

Two ecotypes were used for the dormancy tests: Colombia-0 (Col-0) and Wassilewskija (WS). One week before sterilization, seeds were stored in a desiccator containing *Desiccant-Anhydrous Indicating Drierite* (W.A. Hammond Drierite Company, stock #24001). After which, the desiccated seeds were sterilized by agitating in 70% ethanol for 10 minutes, and then thoroughly dried on sterile filter paper in a laminar flow hood (Figure 1A). The dry sterile seeds were then stored at 4°C in sterile, screw-top microcentrifuge tubes (Figure 1B). A planting protocol is shown in Figure 1; a small amount (50-100 seeds; just enough to plant a few plates at a time) of dry sterile seeds are suspended in sterile water and quickly dispensed onto the surface of solid media (e.g., 0.5% MS solidified with 0.5% Phytigel) using a sterile pipette (Figure 1C). Typically, 12- 20 seeds are dispensed across the top of a single plate. A depth of 40-60 mL of Phytigel media is ideal to support growth of up to 20 arabidopsis seedlings for at least two weeks. The plates are then sealed with porous surgical Micropore® (3M) tape (Figure 1D). The prepared plates are then immediately wrapped in Duvetyne Black-Out Fabric (Seattle Fabrics) to create a light-tight packet (Figure 2). Each step needs to be completed quickly, so that the sequence from the time a seed comes into contact with water or the moist surface of the plate, and then secured in a dark environment, is completed in less than 10 minutes. We typically plant four plates from each

small aliquot of water suspended seed, and use teams of two people (one person to plant and seal the plate in the laminar flow hood, and the other to wrap the plate in Duvetyne). The Duvetyne cloth procedure illustrated in Figure 2 covers

every surface of the square Petri plate by at least two layers of cloth, and creates a light-tight, breathable package that can be stored at room temperature for several weeks.

Figure 1. Dry sterilization and planting for dormant plates. A) Sterilized seeds drying on filter paper in a laminar flow hood. B) Collection of sterilized seeds into sterile, screw-top microcentrifuge tubes, which will be stored at 4°C until use. C) Plating sterilized seeds suspended in water across the top of a single plate with a sterile, disposable pipette. D) Plates are sealed with porous, surgical Micropore® tape and labeled.

Figure 2. Securing plates in a light-tight configuration with Duvetyne Black-Out fabric. A) A survey photograph is taken to show that the configuration of the outer label (top left of image) matches the genotype label on the plate. The plate is then wrapped by first aligning to the bottom of the cloth as shown in (B), then the plate is rolled up in the cloth (C and D), and then the sides are folded in and secured with the outer label (E). The grid shown supporting the plate and cloth in the figure is divided into one inch squares.

The Dormancy Breaking Test

Two sets of plates with dormant WS or Col-0 seeds were prepared, wrapped in black cloth, and left for two, four, or six weeks at room temperature (Figures 3-4). On the respective day, the plates were unwrapped and inspected for

dormancy breakage. After inspection, they were transferred to a growth room and then evaluated for germination to ensure that the seeds remained viable. Plates were scored for the percentage of seed that held dormancy and subsequently germinated (Figure 5).

Figure 3. Dormancy test using Wassilewskija (WS) ecotype seeds. A) Whole plate view of dormant WS seeds after they were stored for 2, 4, and 6 weeks. All seeds remained dormant. B) Close-up view of the selected seeds corresponding to those boxed in red, in 3A. C) Exact plates from 3A that have been exposed to the light and allowed to germinate to show seed viability.

Figure 4. Dormancy test using Col-0 ecotype seeds. A) Whole plate view of dormant Col-0 seeds after they were stored for 2, 4, and 6 weeks. All seeds remained dormant. **B)** Close-up view of the selected seeds corresponding to those boxed in red, in 3A. **C)** Exact plates from 3A that have been exposed to the light and allowed to germinate to show seed viability.

The Illumination Effects on Seed Dormancy Breakage

Two methods of preparing dormant WS plates were used in the illumination test. The first set of plates were planted with the dry sterilized WS seeds and exposed to far-red light ($3.2 \mu\text{mol m}^{-2} \text{s}^{-1}$) for 10 min (Nakashima *et al.*, 2014), and were then wrapped in Duvetyne cloth and stored at 4°C . In the second set of plates, dry sterilized WS seeds were prepared as described above, wrapped in Duvetyne black out-cloth, and stored at room

temperature. After one week, both sets of plates exposed to ambient room lighting ($\sim 100 \mu\text{mol m}^{-2} \text{s}^{-1}$) for 30 seconds to inspect for contamination and germination, and then re-wrapped and stored at either 4°C , or at room temperature (respectively) for three additional weeks. After 4 total weeks from planting, plates from both preparation methods were scored for dormancy breakage, and then subsequently evaluated for seed viability by transferring to growth chamber (Figure 6).

Figure 5. Dormancy and germination (viability) rates of WS and Col-0 and ecotype seeds. **A)** Dormancy (red) and germination (blue) rates of WS seeds after they were stored for 2, 4, and 6 weeks. In WS ecotype, dormancy rates were > 98% and germination rates were > 94%. **B)** Dormancy (red) and germination (blue) rates of Col-0 seeds after they were stored for 2, 4, and 6 weeks. Dormancy rates for Col-0 were > 95% and germination rates were > 93%. For WS, there were between three and six replicates of each time point. Error bars are provided for each set, all standard deviations were less than 0.07. For Col-0, there were four replicates of each time point. Error bars are provided for each set, all standard deviations were less than 0.06.

Figure 6. Visual inspection of dormant, Duvetyne-wrapped plates: **A,B,C)** Plates with WS seeds were treated with Far-red light and stored at 4°C for 1 week before being unwrapped and exposed to 30 seconds of ambient room lighting, and then re-wrapped for storage at 4°C for 4 weeks. The full view of the plate three weeks after visual inspection is shown in (A), and an enlargement of the seed row is shown in (B). **C)** Exact plate from 5A that has been exposed to the light and allowed to germinate to show seed viability. **D,E,F)** Plates with WS seeds were kept at room temperature for 1 week before being unwrapped and exposed to 30 seconds of ambient room lighting, and re-wrapped for storage at room temperature for 4 weeks. The full view of the plate three weeks after visual inspection is shown in (D), and an enlargement of the seed row is shown in (E). **D)** Exact plate from 5A that has been exposed to the light and allowed to germinate to show seed viability.

Figure 7. Actual TAGES flight experiment on-board the ISS, documenting the successful application of this technique in delivering dormant arabidopsis seeds on-orbit. A) Visual inspection of dormant plate 4 (1B49137TK) run 1B showing dormant seeds, and no contamination. B) Identical plate 4 in figure 6B being harvested on ISS after 12 days of growth. C) Wrapped TAGES plate (3A19363TK). D) Identical plate in figure 6C shows dormant seeds, unwrapped and light-stimulated for germination onboard the ISS. The image was taken with the ABRIS/GIS hardware. E,F) Gradual growth of the plant from once dormant seed shows the viability of the seed.

RESULTS AND DISCUSSION

Arabidopsis ecotypes Col-0 and WS were evaluated for their ability to be kept in a dormant, yet viable state for greater than a month. Both ecotypes held dormancy and remained viable for six weeks (Figures 3, 4, and 5). Virtually all (> 98%) of the WS seeds remained dormant at room temperature for up to six weeks, and had a viability rate of > 95% (Figures 3 and 5). Of the Col-0 plates subjected to the same regimen, > 97% remained dormant, and then had an average viability rate of > 93% (Figures 4-5). However, although the Col-0 seeds had a high viability rate, the seeds that germinated after being dormant for six weeks were somewhat smaller than those that germinated after four weeks of dormancy (Figure 4).

Due to the nature of spaceflight experiments, it is crucial to be able to inspect the quality of the prepared dormant plates before they are committed to an experiment. This step minimizes the possibility of launching plates containing seeds that have broken germination, and that may have picked up contamination during the preparations. Since the preparations of plates for spaceflight and other remote deployment experiments typically include two to three times as many plates as will actually be deployed, imperfect plates can be excluded from the set turned over for launch.

Both approaches for creating dormant arabidopsis plates discussed here can accommodate a brief (less than 30 seconds) inspection in normal room lighting (less than 180 $\mu\text{moles m}^{-1}\text{s}^{-1}$) prior to deployment. A comparison of plates prepared by dry sterilization and dark, ambient (20-28°C) temperature storage, and those treated with far-red light treatment and 4°C storage (after the method of Nakashima *et al.*, 2014), is shown in Figure 5. The seeds from both methods remained dormant for three additional weeks after being exposed to ambient room light for 30 seconds one week after they were prepared. Further, greater than 95% of seeds germinated normally when they were finally unwrapped and allowed to grow in standard growth room conditions (Figure 6). These results suggest that opening the light-tight packaging and exposing the dormant plated seeds to ambient light for less than a minute does not drastically affect the seed

dormancy. Thus, the plates can be examined, scored for dormancy, and assured free of contamination before being turned over for a flight experiment, or other application where they can reside at room temperature for an extended period of time before use.

The Transgenic Arabidopsis Gene Expression System (TAGES) experiment carried out in 2009-2010 (Figure 7) illustrates the successful use of this technique to deliver dormant arabidopsis seeds to the ISS for germination on orbit (Paul *et al.*, 2012; Paul *et al.*, 2013b). Dormant seeds wrapped in Duvetyne cloth and stowed under ambient (20-28°C) conditions were transported to the ISS via the space shuttle STS-129, STS-130, and STS-131. Upon arrival they were unwrapped, installed onto the ABRS/GIS hardware, and activated for growth by light stimulation. Multiple plates were used in this experiment, all of which illustrates the successful maintenance of seed dormancy and the viability of these dormant seeds (Figure 7).

CONCLUSIONS

Accurate characterization of plant responses to the spaceflight environment relies heavily on proper sample preparation. In this paper we described a convenient technique used to prepare sterile, dormant, and viable seeds that are well-suited for passive stowage and on-orbit activation. The material of choice for constructing the light-tight packet is Duvetyne Black-Out Fabric (Seattle Fabrics). Duvetyne is also fire retardant, and now vetted for use on the ISS. We were also able to demonstrate that dormant seeds prepared in this manner can tolerate short exposures to light in the middle of their dormancy period, which can accommodate visual inspection of the plates before they are deployed for an experiment. This feature enables investigators to check their dormant experimental plates for contamination or germinated seed, which in turn allows for the removal of compromised plates from the experimental set.

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