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Polyethersulfone (PES) Membrane on Agar Plates as a Plant Growth Platform for Spaceflight

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Abstract

Plant biology experiments in microgravity face many challenges, among which are the constraints of the growth platforms available on the International Space Station (ISS). Protocols for preservation and sample return to Earth often limit efficient dissection of seedlings for downstream tissue-specific analysis. The Advanced Plant Experiment (APEx)-07 spaceflight experiment required a large quantity of dissectible, well-preserved seedlings suitable for omics analysis. During preflight tests, protocols were developed for using an agar-polyethersulfone (PES) membrane platform for seedling growth that allowed for seedling germination and growth aboard the ISS and rapid freezing to provide intact seedlings for dissection and extraction of high-quality DNA, RNA, and protein. Each component of the growth setup was carefully examined: membrane color, hydration and growth substrate, capacity for delayed germination, growth duration, harvest approach, and preservation pipelines were all individually optimized. Sterilized Arabidopsis seeds were adhered to PES membrane with guar gum. Membranes were laid onto 0.8% agar containing 0.5x Murashige and Skoog (MS) in 10 cm square Petri dishes and held at 4 °C until the experiment was actuated by placing the Petri dishes at room temperature. Seedlings were grown vertically for 12 days. PES membranes were removed from the agar, placed in the Petri dish lid, wrapped in foil, and frozen at –80 °C. Seedlings were dissected into roots and shoots and provided high-quality DNA, RNA, and protein. The system is simple, potentially adaptable for seedlings of multiple species, scalable and cost effective, and offers added versatility to existing ISS plant growth capabilities.

Keywords

Spaceflight • advanced plant habitat • microgravity

Introduction

Plant experiments in microgravity are limited by the capabilities of the growth hardware available for use by the research community aboard the International Space Station (ISS). New and innovative experiments often require adapting existing flight platforms to provide new plant growth capabilities for microgravity research. Advanced Plant Experiment (APEx)-07 required a large quantity of Arabidopsis seedlings to be frozen for return from orbit for tissue-specific omics analysis. The experimental parameters required a system in which seedlings could grow for > 10 days, be frozen, remain sufficiently intact to undergo dissection into root and shoot tissues, and adequately preserved to yield high-quality RNA and protein. To this end, a method was developed adapting polyethersulfone (PES) membrane as a growth substrate on agar in 10 cm square Petri dishes for use in NASA's Veggie units aboard the ISS (Figure 1).

PES is a biologically inert, thermostable, hydrophilic polymer with a proven record as a plant growth substrate in microgravity. The European Modular Cultivation System (EMCS), which was in service on the ISS for over a decade and retired in 2018, used PES membrane atop hydrated blotter paper as a seedling growth system. The EMCS PES system facilitated many successful plant experiments during its tenure on orbit (Correll et al., 2005; Millar et al., 2010; Kittang et al., 2014; Mazars et al., 2014; Vandenbrink et al., 2016; Herranz et al., 2019; Sheppard et al., 2021; Villacampa et al., 2021; Meyers et al., 2022). The strategy outlined here draws from those methods while bringing new capabilities to the complement of growth platforms presently available for use aboard the ISS.

Two Veggie units are currently aboard the ISS, each can accommodate up to 30 10-cm round or square Petri dishes placed vertically in a test tube rack (Figure 1). Petri dishes have been used previously in Veggie (Califar et al., 2020; Paul et al., 2021) and the retired Advanced Biological Research System (Paul et al., 2012), and can also be used in Spectrum (www.nasa.gov/mission_pages/station/research). However, in these or previous experiments, seedlings were grown on agar. While nutrient agar has proven to be an effective growth substrate in microgravity as well as on Earth, agar presents limitations that precluded its use with the APEx-07 experiment. Plants

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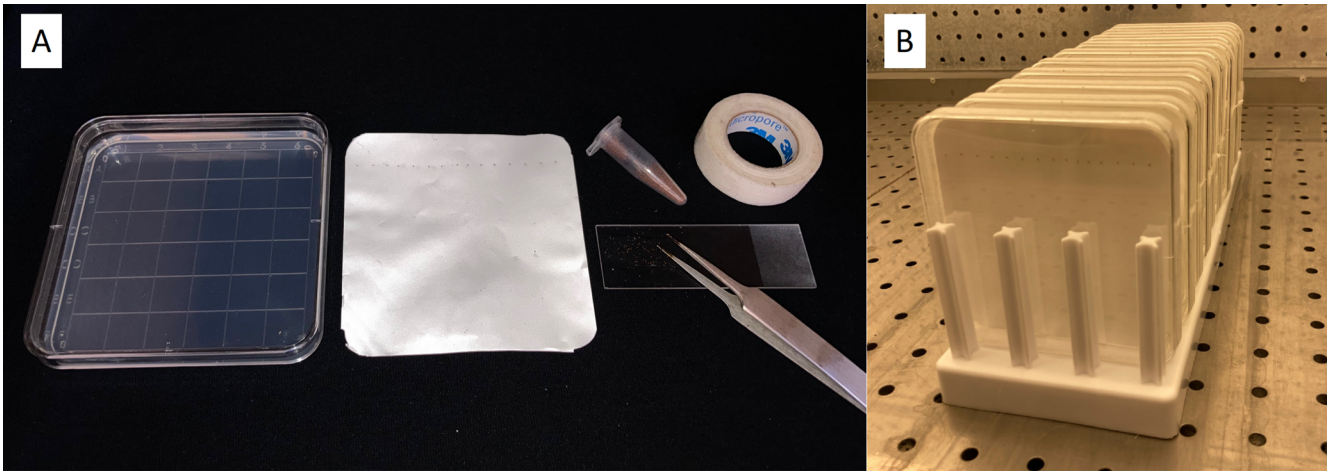

Figure 1. Setup for APEx-07. (A) Plate components from left to right: agar plate, PES membrane, Arabidopsis seed with guar gum, micropore tape. (B) Plates growing vertically in rack.

grown on agar aboard the ISS are generally collected as whole seedlings, placed in fixative using the Kennedy Space Center Fixation Tubes (KFTs), frozen, and returned to Earth (Paul et al. 2005; Hoson 2013; Ferl 2016). The dissection of seedlings into roots and shoots is often not feasible on orbit and requires twice the number of KFTs for sample return. The dissection of whole seedlings returned in KFTs can be labor intensive, especially at high volumes, and fixatives such as RNAlater may also affect downstream analysis (Kruse et al., 2017). Freezing plants directly on agar impedes efficient root collection, as the agar needs to be at least partially thawed before harvest. Here, we developed methods to allow for seedling germination, growth, and preservation aboard the ISS to provide intact seedlings for dissection and extraction of high-quality DNA, RNA, and protein. The approach is versatile, scalable, and cost-effective.

Materials and Methods

Seed sterilization and seeding of membranes

Methods adapted from Meyers et al. (2021). Arabidopsis seeds were sterilized with two 5-minute washes of 70% EtOH + Triton X-100 (1 drop Triton X-100 per 100mL EtOH) followed by a 1-minute wash of 95% EtOH then allowed to dry. PES membrane was sterilized by autoclave. Seeds were then glued to PES membrane using guar gum. A few drops of sterile 1% guar (w/v in H_2O) were placed onto a sterile microscope slide, and several Arabidopsis seeds were deposited into the guar. Sterile forceps were used to individually pick up seeds from guar and deposit them onto PES membrane in a straight, horizontal line approximately 5 cm from the top edge of the membrane. Fifteen seeds were adhered to each membrane. Before the guar solidified, each membrane was viewed under a dissecting microscope to facilitate orientation of each Arabidopsis seed so the micropyle was directed toward the lower edge of the plate. Seeded membranes were kept in a dry, sterile environment until they were needed.

Plate assembly for growth

Sterile 10 cm square Petri dishes (Fisher product number FB0875711A) were used for plant growth. Both white (Sterlitech product number PES0453001) and black (Pall Corporation product number S80677) PES membranes were tested in this system. Both colored membranes had a 0.45 µm pore size. PES membrane and blotter paper were cut into 10x10 cm squares to fit the Petri plates. Both were sterilized by autoclave (121 °C for 25 minutes). Plates were assembled in one of two ways for hydration and growth. First, a sterile blotter was placed in the bottom of a Petri dish (Meyers et al., 2021). Seeded PES membranes were laid on top of the filter paper and hydrated with 0.5x MS liquid media. Alternatively, agar plates were prepared by pouring 40 mL of 0.5x MS 0.8% agar into 10 cm square Petri dishes. Special care was given to let the agar solidify and cool adequately to prevent excess condensation. Seeded membranes were then transferred to agar plates and sealed with micropore tape rather than Parafilm to accommodate gas exchange during the extended growth period. Once hydrated, plates were held in the dark at 4 °C until actuation of the experiment.

Germination delay

Plates with seeded, hydrated membranes were placed in the dark at 4 °C for up to 6 weeks. One plate was removed each week to check for potential germination at 4 °C and confirm seed viability. Three plates were removed and checked at

Figure 2. 12-day growth comparison between black and white PES membrane. The more cost-effective white membranes showed comparable growth to black membrane.

T = 5 weeks, and four plates were removed and checked at T = 6 weeks.

Growth conditions

Plates were kept in the dark at 4 °C for 5 days (as would be needed for late load and flight to the ISS) before they were moved to the Veggie growth units for germination and growth. Veggie parameters were set to long day (16h/8h) and light intensity of approximately 200 µmol m^{-2} s^{-1} and ambient temperature (21 °C). Seeds germinated, and seedling were allowed to grow for 12 days prior to harvest. Sixty plates representing 900 seedlings were grown to test scale up of the system.

Agar/membrane root growth comparison

Seeds were added to plates with 0.5x MS agar and plates with white PES membrane on agar. Plates were stratified in the dark at 4 °C for four days. Plates were moved to a growth chamber with 16h/8h light cycle, and root length was measured at 4 days, 8 days, and 12 days.

Harvest & shipping

Membranes were peeled from the plates (seedlings included) using forceps and either 1) placed directly into a foil pouch and frozen or 2) placed in the lid of the Petri dish, wrapped in foil, and frozen. All samples were initially placed in cold bags (–130 °C) then transferred to a –80 °C freezer until shipment. Samples were shipped from Kennedy Space Center to Athens, Ohio, and Raleigh, North Carolina, on dry ice and assessed for intactness upon arrival.

Molecular analysis

RNA quality and yield were measured in a representative subset of samples. To isolate RNA, plates were removed from the freezer, seedlings dissected into root and shoot tissues, tissues ground using a mortar and pestle, and total RNA extracted using the Norgen Plant/Fungi Total RNA Purification Kit (Norgen catalog number 25800).

Results

Each component of the growth setup was carefully examined: membrane color, hydration and growth substrate, growth duration, capacity for delayed germination, harvest approach, and preservation pipelines were all individually optimized. The cost of the black PES (historically used for contrast and image analysis in EMCS) is several times the cost of white PES, so a comparative growth experiment was conducted. Seedling growth was assessed on both black and white PES membrane, and no difference was found in the growth efficiency between the two membrane colors. Additionally, contrast between roots and white membrane was adequate to collect root imaging data (Figure 2).

The EMCS used PES membranes placed on blotter paper and hydrated with 0.5x MS media, but those experiments

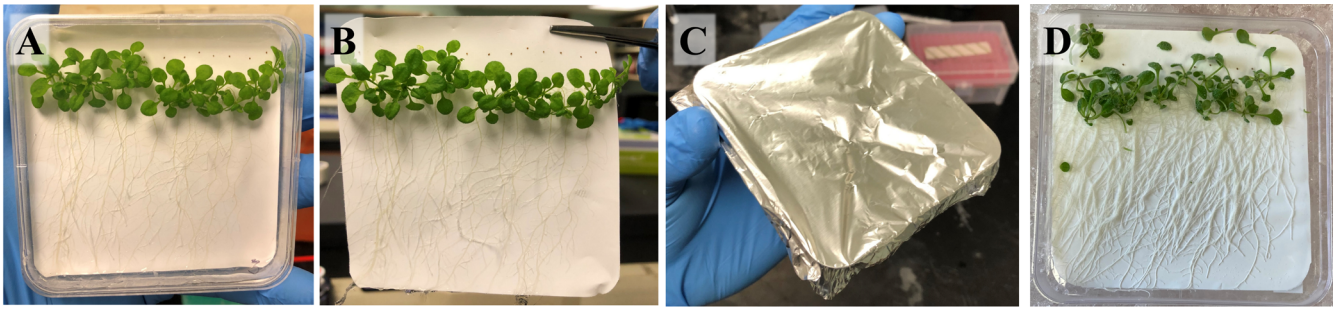

Figure 3. Harvest strategy to maintain intact seedlings. (A) Representative 12-day seedling growth in plate, (B) membrane collected with forceps, (C) membrane transferred to lid of Petri dish and wrapped in foil for freezing and shipping, and (D) a frozen plate returned from low Earth orbit.

were typically five days in duration. Because of the need for a larger quantity of plant material, APEx-07 planned to grow seedlings for longer duration (12–15 days). Thus, a side-by-side comparison of blotter paper versus 0.5x MS agar was conducted to test the ability of each to remain hydrated in a 12-day growth experiment. For this, either 0.5x MS liquid media was added to blotter paper in a plate until saturated or 40 mL of 0.5x MS 0.8% agar was added to each plate. Hydration and growth using 0.5x MS liquid-media-saturated blotter paper was inadequate in maintaining hydration for growth beyond 5 to 6 days even when the plates were sealed with Parafilm. Using 40 mL 0.5x MS 0.8% agar provided adequate hydration for the planned 12-day duration.

To assess differences in growth rates, root lengths were measured in a side-by-side comparison of agar and PES plates at day 4, day 8, and day 12 of growth. The mean root lengths for plants grown on agar were approximately 15–20% longer than those grown on PES membrane across all time points (n = 28). For spaceflight experiments, seeds need to be held in stasis prior to launch and during flight before actuation of the experiment. EMCS had the capability to launch dry membranes with dry seeds adhered, then hydrate the membranes to imbibe the seeds. This was not possible using sealed Petri dishes in Veggie, so germination delay of hydrated seeds was crucial to the success of the system. Feasibility of germination delay was assessed by assembling agar plates with seeded membranes (20 seeds per membrane) and placing them in the dark at 4 °C. Every 7 days, one plate was removed from refrigeration and placed under light at room temperature to check seed viability. For additional replicates at later time points, three plates were checked for viability at T = 5 weeks, and four plates were checked at T = 6 weeks. Of the 240 seeds tested, no seeds showed visible germination at 4 °C during trial, and 100% of seeds germinated when plates were moved to the growth chamber. Thus, germination delay in this system is feasible and had no measurable impact on seed viability.

The return of largely intact seedlings was needed for the downstream tissue dissection, but it was initially unclear how much protection the frozen membranes would require during shipping. In early attempts, individual membranes were placed directly into foil pouches before freezing and shipping; this proved to be inadequate to protect the samples and resulted in highly fragmented seedlings. Ultimately, membranes were harvested from the agar, placed into the lid of their own Petri dish, wrapped with foil, then frozen and shipped on dry ice. The “membrane in lid” strategy provided mostly intact seedlings after shipping (Figure 3).

Success criteria for the APEx-07 experiment included parameters for germination rate, plant growth, contamination, seedling intactness, and RNA quality. Growth metrics were assessed across a 60-plate scale-up (the Experiment Verification Test), and seedling intactness and molecular metrics were measured in a representative subset of the samples. Plates had a 99% germination rate (891/900 seeds germinated) as assessed by visual inspection of photos taken during harvest. No contamination was observed on any of the plates. Growth was deemed adequate if a seedling’s roots were greater than 4 cm. Nearly all seedlings (99.2%) met this metric, with only a few seedlings showing any signs of arrested development. Seedling intactness was deemed acceptable if the seedling could confidently be dissected into root and shoot tissues after shipping. A subset of 10 plates was unwrapped and scored for seedling intactness, and all 10 plates were scored as “excellent” (> 90% of seedlings intact). Total RNA was extracted from four representative root and shoot samples. RNA integrity number (RIN) values for all samples were between 8 and 9.6, and all 260/280 values for RNA were > 2 (Table 1).

Discussion

The trend in falling cost of omics research opens the door for more targeted, tissue-specific investigations of plant response to spaceflight environments (Meyers and Wyatt, 2022). However, tissue specific omics requires the return of mostly intact, high-quality preserved samples from orbit.

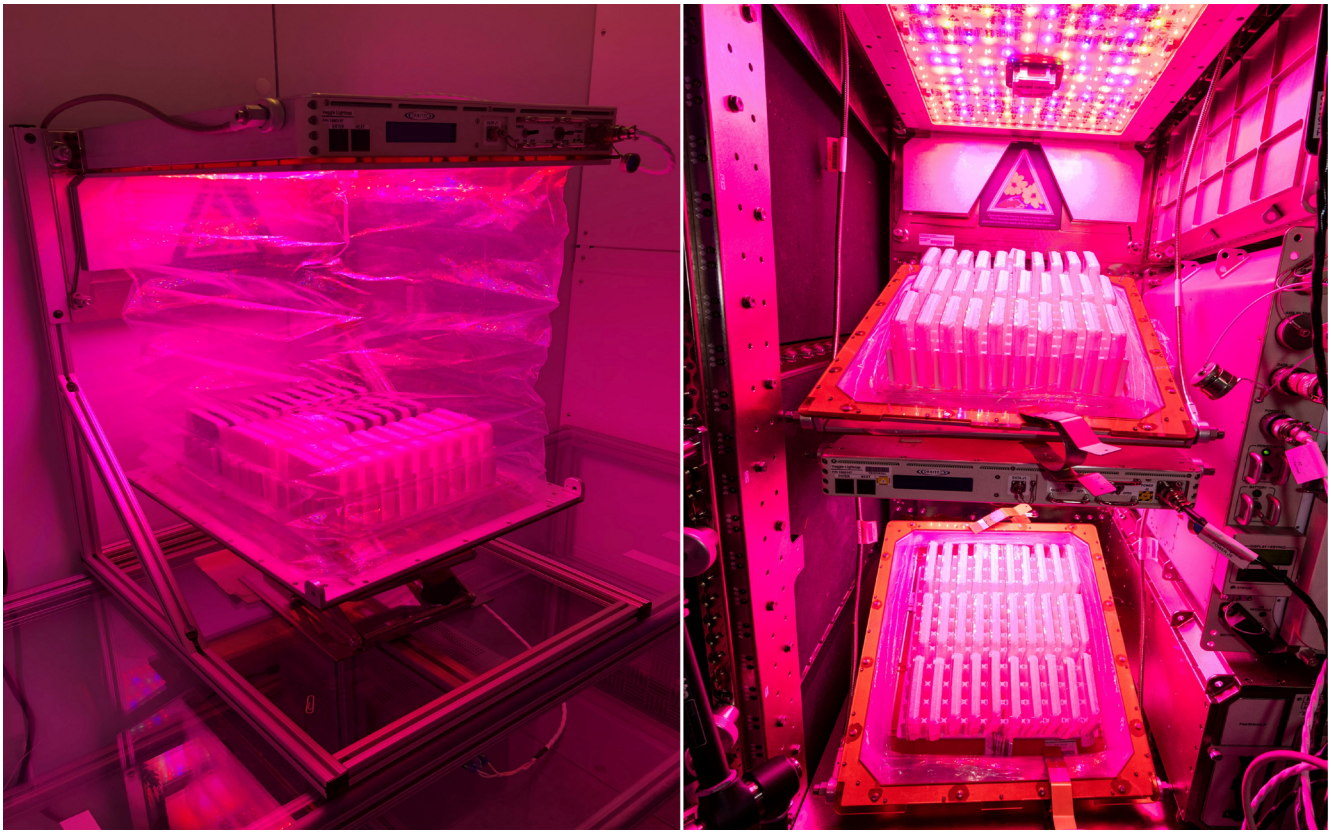

Figure 4. Ground control Veggie unit at Kennedy Space Center (left) and both Veggie units at capacity aboard the ISS (right).

Table 1. Success criteria of EVT samples as measured across 60 plates.

Success Criteria	Goal	Observed	Score
Germination *radical emergence*	>90%	99%	Excellent
Growth *roots > 4cm*	90%	>99%	Excellent
Contamination *Bacterial or fungal growth*	<10%	0%	Excellent
Seedling intactness *Dissectible into roots/shoots*	>90%	100%	Excellent
RNA quality *RIN> 8, 260/280>2*	>75%	100%	Excellent

Existing modalities require sample preservation in RNAlater or by freezing, often making seedling dissection into tissues of interest difficult. Agar plates and PES membrane each have a history of efficacy for plant growth on orbit, and combining the two has now proven to provide added benefits. The system is scalable, versatile, and cost-effective.

Square Petri dishes can be grown in NASA's Veggie chambers. The Veggie units have been in operation on the ISS since 2014 (Zabel et al., 2016). Two chambers are in operation on orbit, with ground control units available at NASA's Kennedy Space Center. The system accommodates a number of growth modalities, including the capacity to hold 30 10-cm square Petri dishes per unit. The light panel of red, blue, and green LEDs can supply > 300 µmol/(m^2*s) of photosynthetic photon flux density, and the units use ambient cabin conditions for temperature and CO_2 (Zabel et al., 2016). Utilizing two Veggie units allows up to 60 Petri dishes of seedlings to be grown simultaneously (Figure 4). Astronaut crew time requirements for this setup are relatively small; installation of 60 plates into Veggie took one astronaut under 30 minutes. Seedling harvest (photographing each plate, collecting the membrane, placing it in the lid, wrapping with foil, and placing it in the freezer) required about 3 hours, with one astronaut harvesting and one astronaut running samples to the freezer. In addition to the Veggie, the same square plate setup could be used with NASA's Spectrum unit (a four-plate fluorescent imager on the ISS), or in the Advanced Plant Habitat. Lastly, PES is sold in circular discs that fit into the 60 mm Petri dishes used in the Biological Research In Cannister (BRIC) and BRIC-LED

systems. The PES agar plate setup could thus be adapted to multiple growth platforms (and possibly multiple plant species) aboard the ISS to return seedling samples fit for dissection, omics analysis, or other physiological measurements.

While testing initial iterations of the PES Petri dish system, it became clear that using black PES membranes for an experiment with a high number of sample replicates would be prohibitively expensive. Black membrane offers greater visual contrast for root imaging, but it is also more than 4x the cost of white membrane (each black membrane costs approximately $17 USD). PES membrane represents the bulk of the expense in this system, so switching to white membrane confers a substantial cost savings. While no formal root imaging was done in the APEx-07 preflight experiments specifically, the white membrane appears to offer adequate contrast for root imaging as well. Buying and cutting membrane from rolls rather than 8" x 10" sheets also minimizes waste of unused membrane. At the time of the experiment, the cost of each fully assembled Petri dish (Petri dish, agar, white PES membrane, seed, guar, MS, and micropore tape) was approximately $4.00 USD.

Conclusions

Spaceflight experiments are often constrained by the limitations conferred by available growth hardware. The simplicity and versatility of the PES-agar plate system opens the door for any number of phenotypic or molecular investigations and invites further innovations to better utilize microgravity growth environments.

Acknowledgments

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