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Research Note • DOI: 10.2478/gsr-2020-0002 GSR • 8 • 2020 • 18–24

Gravitational and Space Research

Growth and Development of Ecotypes of *Arabidopsis thaliana*: Preliminary Experiments to Prepare for a Moon Lander Mission

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Abstract

NASA is planning to launch robotic landers to the Moon as part of the Artemis lunar program. We have proposed sending a greenhouse housed in a 1U CubeSat as part of one of these robotic missions. A major issue with these small landers is the limited power resources that do not allow for a narrow temperature range that we had on previous spaceflight missions with plants. Thus, the goal of this project was to extend this temperature range, allowing for greater flexibility in terms of hardware development for growing plants on the Moon. Our working hypothesis was that a mixture of ecotypes of* Arabidopsis thaliana *from colder and warmer climates would allow us to have successful growth of seedlings. However, our results did not support this hypothesis as a single genotype, Columbia (Col-0), had the best seed germination, growth, and development at the widest temperature range (11–25 °C). Based on results to date, we plan on using the Columbia ecotype, which will allow engineers greater flexibility in designing a thermal system. We plan to establish the parameters of growing plants in the lunar environment, and this goal is important for using plants in a bioregenerative life support system needed for human exploration on the Moon.

Keywords

Arabidopsis thaliana • Artemis Program • CubeSat • Lunar Lander • Plant Morphology • Spaceflight

INTRODUCTION

A new era of lunar exploration has started under the umbrella of the Artemis Program at NASA (Chavers et al., 2019). In the first phase (by 2024), NASA plans to return to the Moon and accomplishes the following tasks: (1) to send robotic landers and payloads to the lunar surface; (2) to assemble the Gateway outpost in lunar orbit; and (3) to resume the first human landings on the surface of the Moon since 1972. NASA's Artemis Program will use a coordinated approach utilizing the resources of the entire agency including NASA centers with a possible focus on landing near the South Pole of the Moon. In the second phase (by 2028), NASA plans to establish a sustained human presence on the Moon. The agency believes that the technologies and instruments developed during Artemis will be useful for future human missions to Mars.

In addition to human spaceflight that is part of Artemis, NASA has developed the Commercial Lunar Payload Services (CLPS) program to send relatively small (<500 kg) robotic landers to the Moon (Chavers et al., 2019). The goals of CLPS include determining whether there are lunar resources for future human missions and conducting science experiments related to Artemis. The first landers under CLPS to land on the Moon are scheduled to be built by Astrobotic (Pittsburgh, PA, USA) and Intuitive Machines (Houston, TX, USA). Based on our previous research on the International Space Station (ISS), we predicted that lunar gravity may be insufficient for normal plant growth and development while Martian gravity is adequate (Kiss et al., 2012; Vandenbrink et al., 2016). These results have profound implications for human exploration plans and the use of a Moon base as a stepping stone to Mars.

We have proposed a plant biology experiment on one of the early CLPS lunar landers that would be in a greenhouse housed in a CubeSat system. Thus, *Arabidopsis thaliana* (L.), a widespread worldwide species, would be grown in a previously designed 1U CubeSat habitat system for plant growth (Kitto et al., 2020). CubeSats are small devices developed for space research and are composed of multiples of 10´10´11 cm (1U) cubic units (Swartwout, 2013). Recently, an experiment with a prototype of this plant growth CubeSat was performed on the ISS (Kitto et al., 2020). Although there were several technical issues with this untended payload, seedlings of *A. thaliana* were successfully grown.

The first CLPS lunar landers have severe restrictions on power and mass, and mission resources available for biological experiments are very limited. In designing experiments with CubeSat on the Moon, a broad spectrum of temperature

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tolerance needs to be considered for growing *Arabidopsis* plants since strict temperature control requires major power resources. The surface temperature on the Moon can vary from −173 °C to +127 °C (Kim, 2020) depending on whether it is lunar day or night (each is 13.5 Earth days). Thus, it will be difficult to have multiple heaters and coolers for these experiments, so we need to develop strategies for the widest temperature range possible for growing plants.

This widest temperature range is in contrast to our previous experiments conducted with *Arabidopsis* seedlings on the Space Shuttle and ISS (Kiss, 2015). In these previous experiments, our planned temperature range was 20–25 °C with the optimum being 22–23 °C. Even with different types of plant growth hardware including Biorack (Katembe et al., 1998; Kiss et al., 1999), Biological Research in Canisters (BRIC) (Millar et al., 2011), and the European Modular Cultivation System (EMCS) (Kiss et al., 2011; Vandenbrink et al., 2016), the optimal temperature goal was easily attained.

In the longer term, ground-based experiments (about 40 days), *A. thaliana* plants were grown at 10–26 °C (Schmuths et al., 2006). However, based on the mission profiles to date, our planned lunar experiment is limited to about 10 days, so we also want to ensure sufficient growth within this time frame.

Fortunately, since *A. thaliana* is a widespread worldwide species and is native to Europe, Asia, and Africa, it can grow in its natural environments under various temperature conditions. Thousands of populations of *A. thaliana* have been collected in the range of this plant, and these ecotypes vary considerably in genotype and phenotype since this species has successfully adapted to many different local environmental conditions (Alonso-Blanco et al., 2016).

Several studies have focused on the physiology and development of natural ecotypes of *A. thaliana* from different latitudinal distributions. For instance, differences in foliar vascular architecture, photosynthetic capacity, and transpiration rate were noted in ecotypes from Italy, Poland, and Sweden (Adams et al., 2016). In a related study, differences in freezing tolerance were found between the Italian and Swedish ecotypes (Park et al., 2018). Similarly, Zhen and Ungerer (2008) tested 71 *Arabidopsis* natural populations originated from 15°N to 60°N latitudinal range and found that northern ecotypes are more tolerant of freezing. In a more extensive series of experiments with over 100 ecotypes from throughout the European range of *Arabidopsis*, seed dormancy and flowering time patterns were correlated with the latitudinal gradient of the plant (Debieu et al., 2013). Interestingly, Li et al. (1998) noted that northern ecotypes tend to have a smaller plant size. In another study, Schmuths et al. (2006) tested for germination and emergence of the radicle at different temperatures of natural ecotypes of *Arabidopsis* from different latitudinal distributions.

Thus, for the reasons for our proposed study, we tested for temperature effects on germination and seedling growth from *Arabidopsis* ecotypes from different latitudes. The parameters used for germination and morphology are standard ones to assess the general health of plants in space missions (Kiss, 2015). We tested in the range of 10–35 °C because the possible temperatures within the contraints of using the robotic landers lie within these ranges.

Thus, our working hypothesis regarding ecotypes of *A. thaliana* is that the optimal temperatures for plant growth would be correlated to latitudinal distributions of this species. In other words, ecotypes from warmer climates like Africa would grow best at warmer temperatures and those from colder regions like Scandinavia would grow best at colder temperatures (Alonso-Blanco et al., 2016). Thus, if we were to use several ecotypes (in one lunar experiment) from wide latitudinal distributions, then we could extend the tolerable temperature range for the robust growth of *Arabidopsis*. This approach would potentially allow us to increase the temperature tolerance range of growing plants in the CubeSat that is part of a proposed CLPS lunar lander mission.

MATERIALS AND METHODS

Plant Material

We studied several natural ecotypes of the plant *A. thaliana* (L.) Heynh. Seeds of these ecotypes were obtained from two sources and are listed in Table 1. Seed stocks of most of the ecotypes used were from the ABRC Arabidopsis Biological Resource Center (https://abrc.osu.edu/). [Note that these seed stocks also are available from the Arabidopsis Information Resource (TAIR) at https://www.arabidopsis.org/]. Additional seed stocks were obtained from Dr. Michael F. Thomashow of Michigan State University, and these lines are described in the studies by Ågren and Schemske (2012), Gehan et al. (2015), and Oakley et al. (2017).

Culture Conditions

Seeds of *A. thaliana* were surface-sterilized with 70% (v/v) of ethanol and a drop of Triton X-100 (200 ml) for 5 min. Seeds were first rinsed in 95% (v/v) of ethanol two times for 1 min each and then rinsed three times with sterilized water for 1 min each. Gridded square Petri dishes 100 ´ 100 ´ 15 mm (Cat. #60872, VWR International, LLC) were filled with 1.2% (w/v) of agar with growth medium, which consisted of one-half-strength Murashige and Skoog salts medium and 1% (w/v) of sucrose at pH 5.5 and is described in the study by Kiss et al. (1997). A layer of sterilized nitrocellulose film (Cat. # V7131, Promega Corp., Madison, WI, USA) was placed on top of the solidified nutrient agar.

Table 1. Key characteristics of the seed stocks of the nine ecotypes used in these studies.

Name	Code	Latitude	Altitude (m)	Mean temperature (°C) Spring/Fall	Origin	Source	Stock #
Tenela	Te-0	N63°	1–100	0–2/7–8	Finland	ABRC	CS1550
Sweden	SW	N62°	NA	NA	Sweden	MSU	**
Osthammar	Ost-1	N61°	1–100	0–2/5–6	Sweden	ABRC	CS1430
Columbia	Col-0	N52°	1–100	15–16/21–22	Poland	MSU	**
Dresden	Dr-0	N51°	100–200	7–8/9–10	Germany	ABRC	CS1114
Italy	IT	N42°	NA	NA	Italy	MSU	**
Coimbra	Co-2	N40°	100–200	11–12/17–18	Portugal	ABRC	CS1086
Martuba	Mt-0	N28°	100–200	15–16/15–16	Libya	ABRC	CS22642
Cape Verde Is	Cvi-0	N16°	1200	NA	Cape Verde	ABRC	CS8580

ABRC, Arabidopsis Biological Resource Center (https://abrc.osu.edu/); MSU, Michigan State University (see Materials and Methods section).

Following surface sterilization, seeds were placed in two rows into each Petri dish. The agar dishes with seeds were then wrapped in Parafilm and left for 72 h at 4°C to stimulate germination. After seed stratification, the plates were placed vertically and continuously illuminated for 10 days with white light fluorescent tubes (120–140 mmol $m^{-2}s^{-1}$) in one growth chamber at 25, 30, or 35°C and in another growth chamber (approximately 240–260 mmol $m^{-2}s^{-1}$) at 11 °C or 15 °C, respectively. For germination tests, the number of seeds used for each ecotype and temperature is shown in Table 2. [However, we believe that it may be difficult to add a stratification step to the lunar experiment given our understanding of mission constraints.]

Image and Data Analyses

Seedlings in Petri dishes were imaged periodically with a Canon EOS Rebel T6 DSLR camera and with a Epson V600 Photo Scanner at termination on the 10th day. Growth and morphometricproperties were measured using the Fiji-win64 software. For each ecotype, the following growth parameters were measured: number of leaves, area of leaves, main root length, number of secondary roots, and the total root length. All analyses were performed with the RGui 64-bit R 3.5.1 for Windows with R Commander package (R Development Core Team, 2008).

RESULTS

Seed germination was assayed in the nine ecotypes of *A. thaliana* from different natural populations at 15, 25, and 35 °C and from four populations at 11 and 30 °C (Tables 1 and 2 and Figure 1), respectively. The highest germination occurred at 15 and 25 °C (Figure 1B,C). Minimal seed germination of 22% or less occurred at 35 °C. Across all temperature conditions studied, the highest seed germination occurred in the Col-0 ecotype. In addition, the SW and Mt-0 ecotypes had good seed germination in the range of 11–30 °C (Figure 1A,D).

Table 2. Number of seedlings grown from each ecotype and used for the temperature experiments.

Seed code	Growth temperature				
	11 °C	15 °C	25 °C	30 °C	35 °C
Te-0	nt	36	24	nt	37
SW	48	36	36	48	36
Ost-1	nt	24	24	nt	20
Col-0	48	36	36	48	36
Dr-0	nt	36	24	nt	36
IT	48	36	33	48	37
Co-2	nt	27	24	nt	24
Mt-0	23	36	36	25	36
Cvi-0	nt	33	24	nt	36

“nt” = not tested because of limited seed stock.

We also examined the morphological features of the plants at different temperature treatments. In terms of leaf development, as with the data on germination, the best results across all ecotypes were at 15 and 25 °C (Figure 2). Thus, the greatest number of leaves (Figure 2A) and the largest leaf area (Figure 2B) occurred when seedlings were incubated at 15 and 25 °C. Similar to the seed germination studies, the best results were obtained with the Col-0 and the Mt-0 ecotypes. At the 30 °C incubation point, only the Col-0, SW, and Mt-0 (but not the IT ecotype) showed limited

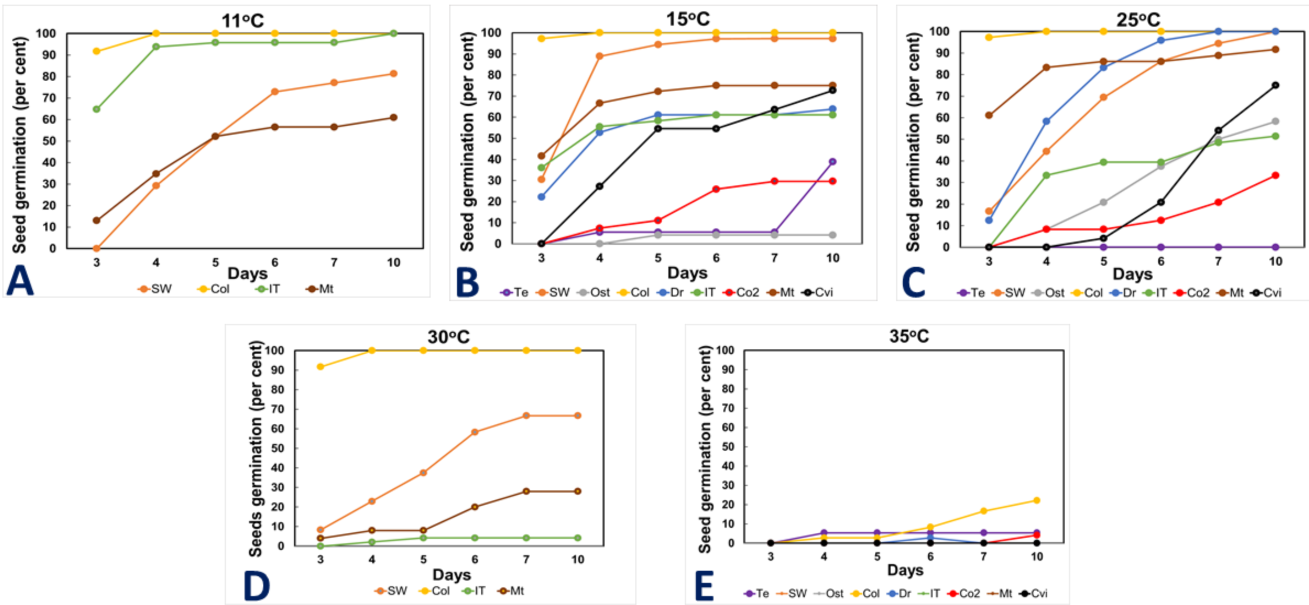

Figure 1. Studies of seed germination of various ecotypes of *Arabidopsis thaliana* from different latitudes. Ecotypes are listed from north (63°N) to south (16°N) at the bottom of each plot. Seeds were incubated at the indicated temperatures in continuous illumination, and seed germination was assayed at days 3–10 after the start of the experiment. Note that the Col-0 ecotype had the highest germination under all conditions studied and nearly 100% in the temperature range of 11–30 °C. The sample sizes are provided in Table 2.

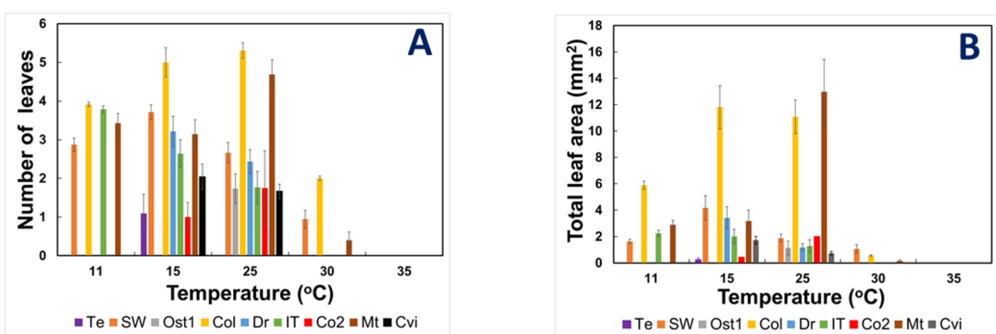

Figure 2. Leaf development assayed by the (A) number of leaves and (B) total area of the leaves in the seedlings at the indicated temperatures in continuous illumination. In terms of the number of leaves (A), the Col-0 ecotype had the greatest number of leaves for all temperatures tested. As indicated by leaf area (B), Col-0 had the largest area when the data were taken together from 11 to 25 °C while the Mt ecotype was an overall second in this latter criterion. The sample sizes are provided in Table 2. Bars indicate SE.

growth of leaves, while there was no growth for any of the ecotypes tested at 35 °C.

The results of the studies of root development (Figure 3) were generally similar to those obtained with investigations of the leaves. In terms of the main root (Figure 3A), the greatest length across all ecotypes was at 15 and 25 °C. The best performers on this parameter again were the Col-0 and the Mt-0 ecotypes. Across all ecotypes, the largest number of secondary roots (Figure 3B) occurred when the seedlings were incubated at 15 and 25 °C, with the Col-0 and the Mt-0 ecotypes exhibiting the greatest number of secondary roots. In terms of the final measurement considered, the length of the total root network (including both the primary and secondary roots; Figure 3C), the results are similar to the previous two parameters. For all three root criteria, there was little or no growth in any of the ecotypes at 30 and 35 °C (Figure 3).

Images of seedlings of the Col-0 (Figure 4) and the Mt-0 (Figure 5) ecotypes illustrate the differences in the

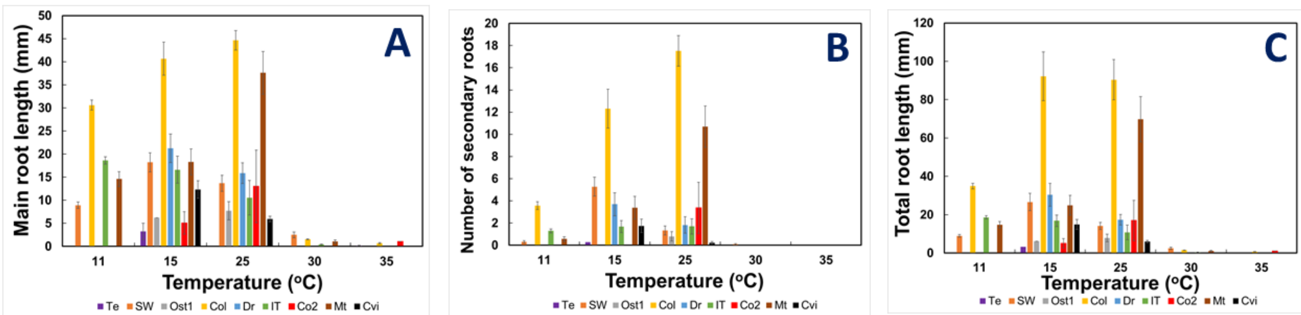

Figure 3. Root development as determined by the (A) main root length, (B) number of secondary roots, and (C) length of the total root network of the seedlings at the indicated temperatures in continuous illumination. Based on these criteria, Col-0 exhibited the most robust growth from 11 to 25 °C. The sample sizes are provided in Table 2. Bars indicate SE.

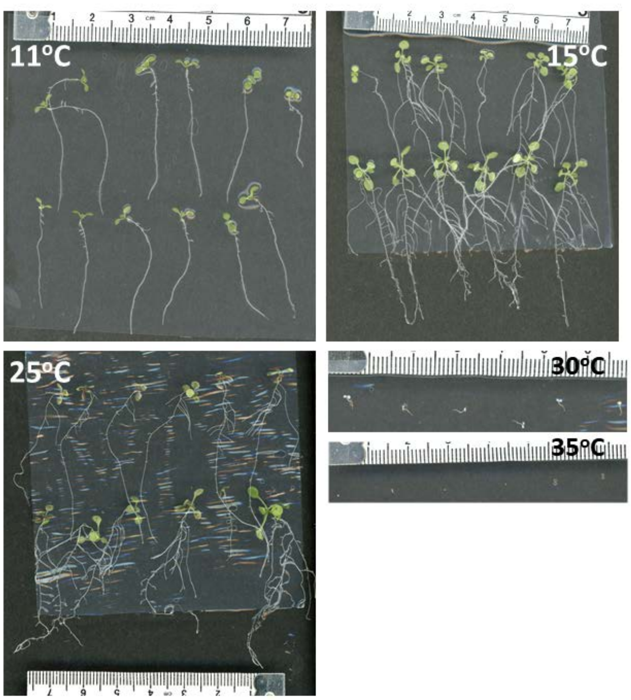

Figure 4. Images of seedlings of the *Col-0 ecotype* of *Arabidopsis thaliana* after 10 days at 11, 15, 25, 30, and 35 °C. While there was good growth at 11 °C, the most robust plants were at incubation at 15–25 °C, and plants at these temperatures had a very extensive secondary root system. There was minimal growth at 30 °C and limited germination at 35 °C. Overall, this ecotype had the most robust growth at a wide temperature range. Note that in some images, there were reflections from the plastic Petri dishes. The large units on the scale reference are in centimeters.

development of the shoot and root systems at the different temperatures studied. Seedlings of the Col-0 show robust growth with a well-developed shoot system and an extensive primary and secondary root networks when incubated at 15 and 25 °C, but there was also good growth at 11 °C (Figure 4). Mt-0 seedlings grow well at 15 °C but have more robust growth at 25 °C (Figure 5). Taken together, when considering seed germination and seedling development, the Col-0 ecotype performed better than the other ecotypes studied at all temperatures tested in these experiments.

DISCUSSION

Studies with Different Ecotypes of *A. thaliana*

Hundreds of ecotypes of *A. thaliana* exist in nature (Alonso-Blanco et al., 2016), so our working plan was to send several ecotypes from varying latitudes on a mission to optimize the success of germination and growth of seedlings and plants in a lunar CubeSat greenhouse (Kitto et al., 2020). We predicted that a mixture of ecotypes from colder (higher latitude) and warmer (lower latitude) environments would allow us to have successful growth of seedlings in our proposed experiment. While other WT strains of *A. thaliana* have been used in previous space flight studies (Vandenbrink and Kiss, 2016), most notably Landsberg (Ler) and Wassilewskija (WS), we chose to focus on strains other than Ler and WS since there were many studies on the broad ecological distribution of these other strains.

However, our initial prediction was not supported by the results obtained in the present study. The Columbia ecotype (Col-0), which can be traced to origins in Poland at 53°N latitude (Fernandez et al., 2018), performed best at a wide range of temperatures from 11 to 30 °C. However, while there was seed germination at 30 °C and even at 35 °C, acceptable growth occurred in the range of 11–25 °C. The next best ecotype in terms of these germination and growth parameters was Mt-0, which is from a Libyan population 28°N latitude (Alonso-Blanco et al., 2016). However, based on the studies to date, Col-0 alone is our preferred choice in these proposed

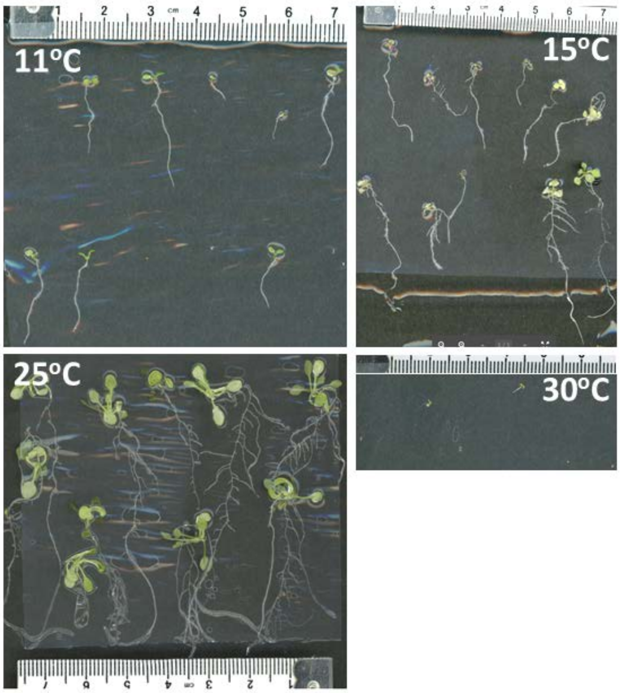

Figure 5. Images of seedlings of the *Mt ecotype* of *Arabidopsis thaliana* after 10 days at 11, 15, 25, 30, and 30 °C. This ecotype was the second-best in overall growth characteristics and did particularly well at 25 °C. There was minimal growth at 30 °C and no germination or growth at 35 °C. Note that in some images, there were reflections from the plastic Petri dishes. The large units on the scale reference are in centimeters.

experiments as it performed much better than all of the other genotypes tested in these studies.

Importance of Optimization of Spaceflight Experiments

Spaceflight opportunities are relatively rare and expensive (Vandenbrink and Kiss, 2016), so it is important to do extensive ground-based testing of many parameters to optimize the flight experiments (Kiss, 2015). In our previous spaceflight experiments, we performed extensive testing to ensure success (Katembe et al., 1998). For example, in the initial EMCS project, we performed extensive ground-based studies as the hardware and the facility were both new. Thus, we tested the effects of storage of seeds on germination and growth as well as cold storage procedures following the termination of the experiment (Kiss et al., 2009). In some of our later EMCS experiments, we improved the lighting and imaging by using infrared illumination to provide high-quality images of the seedlings (Vandenbrink et al., 2019), and ground-based studies were important in identifying and optimizing these parameters (Kiss et al., 2014). Thus, in the present study, we continued using this general approach to determine the optimal temperature range for growing plants in a lunar greenhouse experiment.

CONCLUSIONS

In contrast to our initial working hypothesis of using several ecotypes of *A. thaliana* from populations from varying latitudes to have a wider range of optimal plant growth, we plan to use a single ecotype, Columbia (Col-0), in our proposed lunar studies in a 1U CubeSat greenhouse habitat. Based on the results presented in this study, Col-0 should produce good seed germination and robust plant growth at the range of 11–25 °C. This 14 °C range is broader than the 5 °C (20–25 °C) range used in our previous plant biology space experiments with *Arabidopsis* (Kiss et al., 2014), but it is within a temperature range used in previous ground-based experiments (e.g., Schmuths et al., 2006).

This wider temperature range will give engineers greater flexibility in designing a thermal system in the CubeSat that will be tethered to and acquire power from the robotic lunar lander (Kitto et al., 2020). Our future research will help to establish additional parameters of growing plants in the lunar environment, which will be important in the long-term for using plants as part of a bioregenerative life support system needed for human exploration of the Moon and perhaps Mars (Kiss, 2014).

ACKNOWLEDGMENTS

We thank Ms. Alexandra Settle for her help with seedling growth measurements. We also thank our collaborators from NASA, Drs. Chris McKay and Robert Bowman, for their helpful advice and many thoughtful discussions on lunar biology. Financial support was provided by NASA grant 80NSSC17K0546.

AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

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