Researching Seeds: Films, Sanitation Methods, Microbiological Growth, Viability, and Selection for New Crops

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Researching Seeds: Films, Sanitation Methods, Microbiological Growth, Viability, and Selection for New Crops

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I. Abstract

A major factor in long-term human exploration of the solar system is crop growth in microgravity. Space crops can provide fresh, nutritious food to supplement diets for astronauts. Important factors impacting space plant growth and consumption are water delivery to root zone in microgravity, sanitation methods for microbiological safety, plant responses to light quality/spectrum, and identifying optimal edible plants suitable for growth on the International Space Station (ISS). Astronauts growing their own food on the ISS provides necessary data for crop production for long duration deep space missions. The seed film project can be used in Advanced Plant Habitat and Veggie that are currently being utilized on the ISS.

II. Project 1: Seed Film

The main objective of the seed film is to enable astronauts to handle seeds in microgravity. On-orbit and deep space seed handling in microgravity enables crews to plant crops and microgreens as needed. This new flexibility allows crew members to grow crops on-demand, enables them to grow microgreens, and facilitates crew choice of which crops to grow. Currently, seeds are glued in to APH and Veggie sub-components on Earth, which limits the availability of choice and on-demand gardening. Seed films can be doped with fertilizers, hormones, and beneficial microbes enabling biopriming which is expected to improve space plant health and productivity. The seed film project is currently still in the beginning stages. The current film solution base composition is made in 200-g batches consisting of 18-g of pullulan polymer, 2-g of glycerol, and 180-g of DI water. The polymer takes several hours to fully dissolve and become a clear liquid (*Image 1*). Upon dissolution, it is autoclaved, then cooled under aseptic conditions. The sanitized, room-temperature solution is poured into two square 8" x 8" trays under a laminar flow hood (*Image 2*). Approximately 95-g of solution is poured into each mold resulting in films which are clear and lightweight. The films take about 24 hours to dry and then are carefully removed. The seed film project includes ground testing to verify film sanitization methods, plant germination, and plant growth. Ultimately, the critical ground data will be used to assess flight readiness of the technology as an experiment aboard the International Space Station (ISS).

Image 1. *A film solution clear of pullulan powder clumps after five hours of stirring.*

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Image 2. *Solutions poured into 8" x 8" plates to dry under a laminar flow hood.*

Astronaut safety is of the highest concern. It is important to find the best methods to produce sanitized films containing seeds of various pick-and-eat species. The seed size and geometries of these pick-and-eat crops vary greatly and require seed film processing refinements to produce films which are easily handled, light weight, strong, and with viable seeds which produce crops astronauts can eat. Two types of films will be produced. Microgreen films will contain seeds which are placed in a manner which will produce a bed of microgreen plants in a week or two. Seed bank film is envisioned to store seeds to be used to grow large plants in on-orbit gardens and will have a highly defined placement strategy to enable crew members to easily cut the seeds of interest from a sheet or ribbon and place into the on-orbit garden system for germination and growth. Both film types should be sanitized as a safety precaution.

The preferred method to sanitize the films is to autoclave the film solution prior to casting to kill all biology in the solution. Seeds are sanitized as recommended by staff scientists and film production techniques are performed aseptically in a laminar flow hood. Microbial assessments were used to determine the effectiveness of the autoclave methods. Multiple experiments were performed to test the effectiveness of the autoclaving method. The first autoclave method held the solution for 15 minutes at 120°C and proved to be ineffective since microbial growth was noted on processed TSA and IMA plates. The procedure was updated to use sterilized scalpels for film removal and increased the autoclave dwell time to 35 minutes. The same procedure to assess active microbiology was used, three small pieces from each of the top, middle, and bottom of the dry films were cut and placed on the TSA and IMA plates. The procedure is shown in *Image 3.* For the autoclaved seed film solution 0.05 mL was pipetted onto the TSA and IMA plates for microbial assessment. The results were not ideal with microbial growth found on the sanitized film and solution plates, shown in *Figure 1*. There is a clear distinction between the autoclaved solution film strips and the unsanitized strips (*Images 4* and *5*). The unsanitized film strips had a significant amount of bacterial growth around them. The sanitization method is still under review. The main interest is to have no human or plant pathogens in the films.

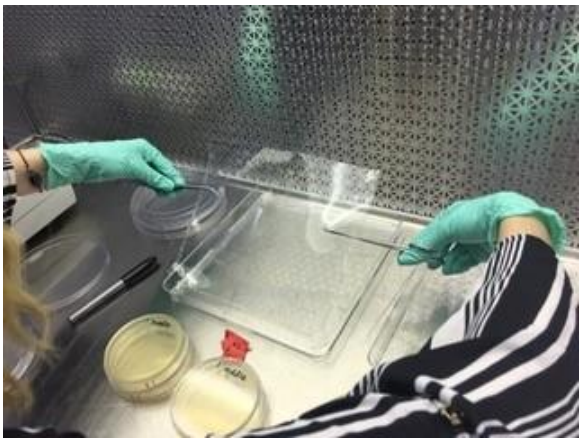

Image 3. *Cutting out the middle strip of an autoclaved seed film.*

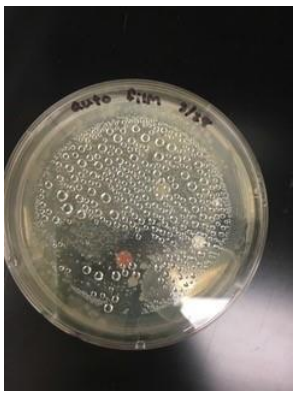

Image 4 (left) and Image 5 (right). *The unsanitized film plate (right) has bacterial growth on the film strips directly, while the autoclaved film strips (left) do not.*

Microbial Growth		
Plate	TSA	IMA
Unsanitized Film	Yes	Yes
Sanitized Film	Yes*	No
Unsanitized Solution	Yes	Yes
Sanitized Solution	No	Yes

Figure 1. * *Bacterial growth not on the films themselves, but spread throughout the plate.*

The next step for the seed films is radiation testing at the NASA Space Radiation Laboratory in early June. Radiation testing requires at least ten seed films with forty seeds in each 5" x 5" section or two films containing 20 seeds each. The resulting films need to have appropriate seed density allowing easy film cutting to cut out select seeds for testing. These radiation test films do not need to be sanitized. A total of 800 Red Romaine lettuce seeds will be used for this project. The films, containing the seeds, will be stored in ten ziplock bags (one or two films in each bag). They will be tested with Silicon, and proton, and mixed beams to determine their susceptibility to radiation in space.

III. Project 2: Seed Sanitation

Veggie can be considered as a simple, low power space garden which "provides edible crops for the crew to have a palatable, nutritious, and safe source of fresh food. It is also a tool to support relaxation and recreation" [1]. Veggie is not restricted to space use, the system can also improve plant production on Earth. Food production and astronaut nutrition are two important facets of Veggie. Astronaut safety is of the highest concern when it comes to consumption of these crops. In order to keep the crops grown on the ISS safe for astronaut consumption, we ensure the seeds do not harbor human pathogens by surface sanitizing methods. Seeds vary in their microbiome, surface morphology, and surface chemistry which complicates seed sanitation methods. One seed sanitation method involves utilizing a hydrochloric acid (HCl) and bleach solution that creates an oxidizing vapor. To begin the procedure, a number of seeds are selected and placed in a 50 mm glass petri dish. A Ball Jar is used with a small 50 mL beaker placed inverted on the inside of the jar. 30 mL of store bought bleach (5.25 wt% hypochlorite solution) is added to each jar. Typically no more than four Mason Jars are used at a time. In a fume hood, 0.5 mL of concentrated HCl is pipetted into the base of the Ball Jar using a 1000 µL pipette, shown in Image 6. The dish containing the seeds is placed on top of the inverted beaker and the lid is sealed. The petri dish is small enough to allow the axidizing vapor to permeate the entire jar. The seeds are left sealed in the jar for an hour. Once the elapsed time is over, the dish is removed from the jar and the seeds are left to outgas overnight under aseptic conditions in a laminar flow hood. Ethanol is used to clean the laminar flow hood surface before transferring the seed dishes. Proper PPE is always used during this procedure and it includes safety goggles, gloves, and a lab coat. A second person always needs to be in the room in case of an emergency from inhalation or a spill occurs.

Image 6. *Pipetting 0.5 mL of HCl into the 30 mL of bleach.*

Image 7. *Germination plate alignment under the lights in reach-in-chamber G.*

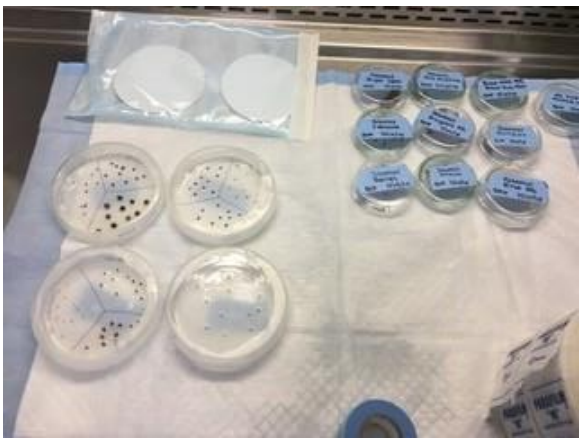

Image 8. *Ten seed types on germination plates.*

After outgassing, the petri dishes containing the seeds are sealed using parafilm and transferred to another laminar flow hood in the microbiological lab. The seeds are then examined for stress response, germination rates, viability, microbiological growth, and molecular data is collected and used to identify any remaining microbiological life. Germination testing is used to gauge success of the sanitization method. A typical germination test involves placing 10 sanitized and 10 unsanitized seeds on autoclaved filter paper in a glass petri dish. The filter paper is first saturated with water and then the dish is tightly sealed with parafilm to keep in the moisture. The germination plates are placed in a reach-in chamber with a day/night cycle lighting system (*Image 7*). The plates are then checked daily and observations are recorded. The plates are re-watered and re-filmed when necessary. Microbial testing involves plating the sanitized and unsanitized seeds on Tryptic Soy Agar (TSA) and Inhibitory Mold Agar (IMA) plates. The TSA plates allow for bacterial growth, while the IMA plates contain antibiotics that allow for solely fungal growth. Five sanitized seeds are placed on a TSA plate and five on an IMA plate under aseptic conditions. Two plates are also prepared in the same manner with unsanitized seeds. All plates are then placed in a 35°C incubator. The plates are checked daily for growth and observations are recorded.

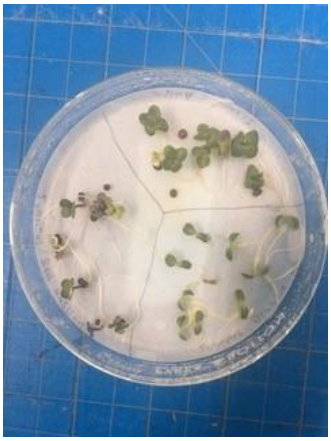

Image 9. *Germinated seeds.*

New crop selection activities involved ten different kinds of seeds from three different vendors (Image 8). All seeds require a seed surface sanitization method which renders the microbiology inactive and the seed viable. To start the previously described fuming method was tested which used 30 mL bleach / 0.5 mL HCl at a one hour. Microbial testing showed that by day eight, see *Figure 2*, the seed sanitation procedure proved successful for most of the seeds. Their germination from days four and five are shown in *Figure 3*. Germination progress can be seen in Image 9 of Johnny's Red Russian, Amara, and Dragoon. Ultimately, Wasabi Mustard, Extra Dwarf Pak Choi, Red Russian Kale, and Dragoon Lettuce were determined to be the best candidates for the new crop selection, largely based on this testing.

Seed Type	TSA Sanitized	TSA Unsanitized	IMA Sanitized	IMA Unsanitized
Johnny's Toscano	2/5*	3/5	0/5	0/5
Johnny's Shungiku	Contamination	5/5	1/5*	5/5
Johnny's Dragoon OG	0/5	5/5	0/5	5/5
Johnny's Amara	0/5	5/5	0/5	5/5
Johnny's Bright Lights	0/5	5/5	0/5	5/5
Johnny's Sorrel	0/5	0/5	0/5	0/5
Bakers Creek Pak Choy	0/5	5/5	0/5	2/5
Johnny's Eros OG	0/5	4/5	0/5	3/5
MV Seeds Wasabi	0/5	5/5	0/5	0/5
Johnny's Red Russian	0/5	2/5	0/5	0/5

Figure 2. Microbiological Growth checked on 3/28/18 (Day 8) **these growths appear to be caused by contamination, potentially from the handling of the plates. They are not growths directly on the seeds, but affect/cover the number of seeds listed.*

Seed Type	Day 4	Day 5
Johnny's Toscano	5/10	5/10
Johnny's Shungiku	8/10	8/10
Johnny's Dragoon OG	10/10	10/10
Johnny's Amara	8/10	8/10
Johnny's Bright Lights	4/10	7/10
Johnny's Sorrel	5/10	5/10
Bakers Creek Pak Choy	10/10	10/10
Johnny's Eros OG	4/10	4/10
MV Seeds Wasabi	9/10	9/10
Johnny's Red Russian	9/10	9/10

Figure 3. *Germination on autoclaved filter paper.*

Multiple seed sanitation experiments were performed on Red Robin tomato seeds to determine the most effective concentrations and time durations. Testing involved varying the amount of HCl in bleach. For example, one experiment increased the quantity of HCl from 0.5 mL to 0.75 mL. Another variable involved dwell time. Fumigation duration was varied between 60 minutes up to 120 minutes. Data was assessed for the tomato seeds resulting microbiological growth and germination rates. The data is shown in *Figure 4* and *Figure 5*. Experiments indicated that 0.5 mL of HCl at two hours as the best procedure for the Totally Tomatoes Red Robin tomatoes for use in Veg-05 SVT. More experiments are in progress to refine the method for the seeds to bed used in Veg-05 EVT. The *Figure 4* table also shows data for Mizuna and Outredgeous sanitation tests for a seed microbiome project.

		Microbiological Growth on Plates		Germination	
Date Checked	Seed Type/Treatment	TSA	IMA	TSA	IMA
February 22nd					
Mizuna	Reimer Sanitized	0/5	0/5	1/5	2/5
	Heirloom Sanitized	0/5	0/5	5/5	5/5
	Johnny's Sanitized	0/5	0/5	3/5	5/5
	Reimer Unsanitized	5/5	1/5	0/5	3/5
	Heirloom Unsanitized	3/5	1/5	5/5	5/5
	Johnny's Unsanitized	3/5	0/5	3/5	4/5
March 28th					
	Totally Tomatoes Sanitized	2/5	1/5	0/5	0/5
	Park Seed Tomatoes Sanitized	0/5	0/5	0/5	0/5
	Tomato Fest Sanitized	1/5	1/5	0/5	0/5
	Totally Tomatoes Unsanitized	not plated	not plated	0/5	0/5
	Park Seed Tomatoes Unsanitized	5/5	5/5	0/5	0/5
	Tomato Fest Unsanitized	5/5	5/5	0/5	0/5
April 2nd & 3rd					
Totally	.75 mL of HCl 1 hr	0/5	0/5	0/5	3/5
Tomatoes	.75 mL of HCl 1.5 hr	0/5	0/5	0/5	2/5
	.75mL of HCl 2 hr	0/5	0/5	0/5	0/5
	.5 mL of HCl 2 hr	0/5	0/5	0/5	0/5
	Unsanitized	5/5	2/5	0/5	2/5

Figure 4. *Microbiological growth and germination on TSA/IMA plates.*

		Germination on Filter Paper				
Date Plated	Seed Type/Treatment	Feb 24th	Feb 27th	Mar 20th	Mar 21st	Mar 22nd
March 30th						
Totally	.75 mL of HCl 1 hr	0/8	1/8	6/8	7/8	7/8
Tomatoes	.75 mL of HCl 1.5 hr	0/10	0/10	0/10	1/10	3/10
	.75mL of HCl 2 hr	0/8	0/8	0/8	0/8	0/8
	.5 mL of HCl 2 hr	0/10	1/10	8/10	8/10	8/10
	Unsanitized	0/10	0/10	5/10	7/10	7/10

Figure 5. *Germination of Totally Tomatoes on autoclaved filter paper.*

IV. Project 3: Microbiological Work for Veggie and Market Produce

Veggie is a plant growth system used to grow fresh crops for scientific experiments and astronaut consumption. Veggie has many important implications for current space exploration and future deep space missions. The system not only provides food, but physiological benefits to the crew members. Veggie provides a close-to-home feel by having plants grow, in space, on the ISS. Microbiological experiments with Veg-04 SVT plant samples, Veggie ponds swabs, and Veg-03 pillow samples are performed. Pillow samples come from Veg-03 A, B, and C. Each of the sets have two flight and two ground samples. For the pillow microbial analysis: tubes for the water, roots, and three soil samples of each pillow are collected. The tube weights are collected first, then the weights of the tubes containing the samples are collected. Each tube needs to be shaken up for two minutes after the weights are recorded. Dilution tubes are set up with the factors of -2, -3, -4, and -5. 1 mL of each sample is pipetted onto an

Escherichia coli 3M Petrifilm and placed in the 50°C incubator. Each sample is plated and spread on TSA/IMA plates. The plates are placed in the 35°C incubator. The microbial colonies are counted on each plate after incubation.

Mary Hummerick's work is focused on comparing the sanitation of market produce and crops grown in our chambers. The purpose of this experiment is similar to the seed sanitation experiments; they are both focused on food safety. Radish, tomato, and mizuna samples are collected from the chambers and prepared for experimentation. Buffer peptone solutions are made and 4.5 mL is pipetted into dilution tubes. The tubes are labeled -2, -3, -4, and -5 for their dilution factors. Plates and 3M Petrifilms are labeled for both clean and unclean samples. Three unclean samples are weighed first and then bagged. The other three samples are cleaned by being shaken up in a plastic bag filled with sterilized water. The cleaning requires three reps of fifteen second intervals for every sample. The clean samples are transferred to a filter paper bag using a sanitized cloth and then weighed. 200 mL of buffer peptone solution is added to each bag (total of 6 samples) using a sterile graduated cylinder. The bags are then placed in the bag mixer for two minutes each. 1 mL is pipetted from the bag samples onto each 3M Petrifilm without creating any bubbles. Bubbles are a sign of growth and should be avoided when covering the films. 0.5 mL is pipetted from the bag samples into the -2 dilution tube, 0.5 mL is then pipetted from the -2 tube to the -3 tube, and so on. 0.05 mL of each sample is pipetted onto the TSA/IMA plates and spread. Once the plates are prepared, they are placed in the 35°C or 50°C incubators. The plates are checked daily for any microbiological growth.

V. Project 4: Harvests

Image 10. *Red Robin tomatoes.*

One of the biggest, and most important, tasks required in the space plant biology department are the plant harvests. They occur every 14, 21, and 28 days. There are different procedures and data collected for each harvest. For the Strategic LED Lighting Effects on Development (SLLED) harvests with Matt Mickens: five anthocyanin measurements per plant, three chlorophyll measurements (averaged) using the SPAD, leaf area and leaf number are collected. Leaves are collected in bags or in tubes that are placed in liquid nitrogen to fast freeze. Plant samples stored in liquid nitrogen are later chemically analyzed. For the ILSRA tomato harvests with Gioia Massa (*Image 10*), leaf area and leaf number were collected. For the New Crop Selection harvests with LaShelle Spencer, the plants would be weighed and bagged. After the final harvest of each round for the New Crop Selection, a blind taste testing is performed to determine the highest and lowest ranked crops in taste-bud appeal. For the ILSRA harvest of Red Robin tomatoes for Matt Romeyn and Gioia Massa: all of the out-growths from the main stem are counted, the plant height and width is measured, the fruit is removed and counted, the ripe and green tomatoes are weighed, and the leaf matter is removed then weighed.

VI. Project 5: Martian Soil Simulant Experiment

A Martian soil simulant experiment is in progress. The growth of "Extra Dwarf" Pak Choi grown in two-inch pots with regolith will be analyzed. The plants will be grown in a chamber using the same conditions as the ISS. The purpose of this experiment is to analyze the toxicity of the plant after being grown in this fine regolith. Regolith is a combination of dust, soil, and bedrock similar to what would be found on Mars surface. It is important to know the effects of the Maritain simulant soil on the plants for future experiments and explorations. Microbial growth, soil and plant toxicity, and seed viability will be tested.

The environmental conditions are:

Temperature – 23°C (constant)
RH – 50% (constant)
CO_2 – 3000 ppm
~PPF – 350 μmol $m^{-2}s^{-1}$

Photoperiod – 16 hours of light, 8 hours of dark. On at 8:00, off at 24:00.

References

[1]Massa, G. D., and Levine, H. G., "Veg-03," *ISS Program Science Office*.