Fiscal Year:	FY 2023	**Task Last Updated:**	FY 03/02/2023
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Project Title:	Dissecting Beneficial Plant-Microbe Interactions and Their Efficacy in the ISS Spaceflight Environment, a Model Study		
Division Name:	Space Biology		
Program/Discipline:			
Program/Discipline--Element/Subdiscipline:			
Joint Agency Name:		**TechPort:**	No
Human Research Program Elements:	None		
Human Research Program Risks:	None		
Space Biology Element:	(1) Cell & Molecular Biology (2) Microbiology (3) Plant Biology		
Space Biology Cross-Element Discipline:	None		
Space Biology Special Category:	None		
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Comments:			
Project Type:	FLIGHT	**Solicitation / Funding Source:**	2018 Space Biology (ROSBio) NNH18ZTT001N-FG. App B: Flight and Ground Space Biology Research
Start Date:	01/01/2020	**End Date:**	12/31/2023
No. of Post Docs:	1	**No. of PhD Degrees:**	
No. of PhD Candidates:		**No. of Master' Degrees:**	
No. of Master's Candidates:		**No. of Bachelor's Degrees:**	1
No. of Bachelor's Candidates:	1	**Monitoring Center:**	NASA KSC
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Flight Program:			
Flight Assignment:	NOTE: End date changed to 12/31/2023 per NSSC information (Ed, 1/15/23)		
Key Personnel Changes/Previous PI:			
COI Name (Institution):	Davin, Laurence Ph.D. (Washington State University, Pullman) Kahn, Michael Ph.D. (Washington State University, Pullman)		
Grant/Contract No.:	80NSSC19K1484		
Performance Goal No.:			
Performance Goal Text:			

Task Description:	Significance of objectives to NASA and this Solicitation: Deep space exploration or extraterrestrial colonization (e.g., Moon or Mars) will require the ability to sustainably produce plants for human/animal consumption, as well as providing aesthetic benefits of plant life to future crews and personnel in extra-terrestrial colonies. One key challenge in spaceflight/microgravity is in overcoming long-standing difficulties in efficaciously providing water and nutrients to germinating and maturing plants. Another important research challenge that has seen little attention is in productively exploiting beneficial plant-microbe interactions in spaceflight/microgravity, particularly for nitrogen (N) fixation. When both challenges are resolved for optimal, productive, and efficacious plant growth in space, this will provide the exciting opportunity to recycle organically bound carbon (C) and N that was sequestered in these plants. Through subsequent recycling of those organics (e.g., derived from human and animal consumption waste and from unused plant parts), this will help enable sustainable plant growth over multiple generations. Another benefit of studying beneficial plant microbe interactions is at the fundamental science level, i.e., by gaining much improved understanding of how the spaceflight/microgravity environment affects this important physiological process. Central objectives of proposed research: Complementary purposes of our 2 Specific Aims are to initially dissect, understand, and optimize plant growth/development in spaceflight/microgravity via exploiting beneficial plant-microbe interactions. Then to ultimately recycle organic C and N from them suitable for subsequent multiple plant generations. To do this, we will use model Medicago plant species (e.g., alfalfa), and its beneficial bacterial symbiont, which together can potentially displace the need for N-containing fertilizer in spaceflight/microgravity. Specific Aims: 1. Comprehensively compare and contrast efficacy of beneficial symbiotic plant-microbe interactions between Medicago and Sinorhizobium growing on the International Space Station (ISS) and on Earth (NASA Kennedy Space Center/KSC), including understanding changes occurring at the molecular level; 2. Compare and contrast ease of biodegradation of the ISS and Earth grown Medicago plant material, in order to assess whether there are any differences in the ability to recycle C, N, etc. for multiple generations of plant growth/development. Justification for this work is threefold: The first is to demonstrate that beneficial plant microbe interactions during N-fixation can be efficaciously achieved in spaceflight/microgravity. The second is to gain a fundamental understanding of spaceflight/microgravity environment effects on these beneficial plant microbe interactions, and their potential usage for deep space exploration and colonization. The third is to demonstrate that organic C and N can be facilely recycled to support multiple generations of plant growth in space. Methods/techniques: During growth, FluorPen and plant size measurements will be carried out to assess comparative N-fixation efficacy for each condition, both on the ISS and on Earth. Tissues (leaf, stem, and root) from the ISS and Earth control will be collected after ca. 6-8 weeks growth, frozen (-160°C). They will be subjected to transcriptomic and metabolomic (including amino acid) analyses; the microbiomes present in aerial/underground tissues will be determined. The multi-omics approaches employed are as for our Arabidopsis study. Medicago plant material, from the ISS and ground control, will also be subjected to biodegradation to establish whether there are any differences in N-mineralization (for recycling) in spaceflight/microgravity or ground control tissues.
Rationale for HRP Directed Research:	
Research Impact/Earth Benefits:	Among the benefits on Earth envisaged: improving our knowledge of N-fixing process and the symbiosis between Medicago and Sinorhizobium, and determining optimal lignin contents for space and Earth will be very instructive, as will the recycling C/N capabilities for both wild type and genetically modified plant lines. Demonstrating this in space is also a very effective means of demonstrating to aspiring young scientists (including Middle and High School students) and others of the importance of plant life, of N-fixation, and of C/N recycling in a sustainable manner.
	1.1. Alfalfa-Sinorhizobium symbiosis in Passive Orbital Nutrient Delivery System (PONDS) and APEX (Advanced Plant Experiment) Systems – initial experiments As described in the previous progress report, growth of alfalfa (M. sativa), together with its symbiont S. meliloti, was quite extensively evaluated in both PONDS and APEX systems, using the alfalfa Ladak cultivar and S. meliloti, as well as with commercial low lignin and normal (wild type, WT) lignin level alfalfa lines. In our ground-based studies extending until early Spring 2022, conditions were identified for satisfactory symbiotic growth/development of alfalfa with S. meliloti using the PONDS system, and we were thus poised to advance to the Science Verification Test (SVT) phase. As an example of our progress, since carbon dioxide (CO2) levels on the International Space Station (ISS) average 3,500 ppm with relative humidity (RH) levels consistently at 45-50%, we next evaluated alfalfa growth conditions in combination with previously optimized lighting levels determined at “normal” CO2 and lower ambient humidity. Alfalfa plants were grown in magenta containers. After 6 weeks, alfalfa plants grown under 3,500 ppm CO2/45% RH conditions grew significantly taller, with larger diameter and denser stems than those of plants grown in a 410 ppm CO2 atmosphere and 30% RH. These results indicated that the environment on board the ISS was suitable for growing alfalfa, at least with respect to RH and CO2 levels. However, our work using PONDS had to be abruptly abandoned as an unrelated (to us) PONDS test study on the ISS failed in early 2022; this information being provided by NASA Kennedy Space Center (KSC) research personnel that were associated with the PONDS ISS test. We were also informed by NASA personnel that the PONDS system would now no longer be available for our proposed ISS study. (More recently, while a modified PONDS system re-flight has been rescheduled for further ISS hardware testing in 2023, we were advised by NASA to develop other systems for our proposed ISS study.) 1.2. Re-focus on APEX plant growth chambers for ISS This unexpectedly brought us back to further development of the APEX system to meet our ISS study goals which had previously been sub-optimal using the APEX equipment. The timeframe from Spring 2022 to December 2022 was thus focused upon establishing conditions that would allow for efficient symbiosis and alfalfa-root nodule formation, as well as for satisfactory growth of low and normal lignin level lines, in the APEX system. (An alternate configuration of

Task Progress:	APEX was thus designed.) Experiments with this APEX configuration resulted in somewhat better growth of alfalfa containing a developed root system with N-fixing nodules, although the plants did not grow to the same levels obtained when N was added as a supplement. Since then, ground testing of alfalfa growth conditions in APEX hardware has culminated in near full completion of our final definition experiment leading up to SVT (i.e., which focuses both on satisfactory symbiosis as well as in generating sufficient alfalfa plant tissue for metabolomics, transcriptomics, lignin and lignin biodegradation analyses, etc.). To do this, twenty-four APEX Growth Chambers (AGCs) were assembled. In one experiment, 6 AGCs contained low-lignin alfalfa seed, and 6 AGCs contained a WT alfalfa line with WT lignin levels. All AGCs in that experiment had an added nitrogen source. The 12 AGCs containing these seeds were placed in a surrogate VEGGIE growth chamber, with the experiment initiated by adding a nutrient solution followed by water as needed for the duration of the plant growth. After 37 days of growth, both alfalfa lines grew as expected. In the S. meliloti-alfalfa symbiosis experiment, the other 12 AGCs contained WT alfalfa cv. Ladak seed. To test the effectiveness of the symbiosis, 6 AGCs contained S. meliloti inoculum, 4 AGCs contained an added the nitrogen source (positive controls), and 2 AGCs (negative controls) did not contain any nitrogen or S. meliloti. The 12 AGCs were also placed in a surrogate VEGGIE growth chamber. Again, plant growth was initiated by adding a nutrient solution followed by water as needed for duration of the experiment. After 37 days of growth, alfalfa inoculated with S. meliloti showed reasonably good plant growth, i.e., as expected in an effective symbiosis, whereas the controls showed either good growth (positive controls) or poor growth (negative controls). 1.3. Lignin-reduced alfalfa lines As described previously, application of CRISPR/Cas9 was carried out to generate lignin-reduced alfalfa lines, as well as growing the same low-lignin and normal alfalfa seed described above. All lines produced were grown to assess their relative growth/development and lignin-reduction characteristics. 1.3.1. Genetic engineering for lignin-reduced alfalfa Our previous studies involving downregulation of arogenate dehydratase (ADT) genes in Arabidopsis thaliana resulted in plants with reduced lignin levels. Initially, as described earlier, we obtained ADT homologs from M. truncatula followed by corresponding genes from cDNA preparations of M. sativa variety “Ladak” 4-week-old leaf tissue total RNA. The CRISPR/Cas9 gene-editing approach was next selected to disable ADT genes, potentially allowing for multiple gene knock-out targets in a single experiment. Currently, we are screening regenerated transgenic plants to establish mutations in targeted genes. Transgenic CRISPR/Cas9 plants that showed promising results had sequence-confirmed CRISPR/Cas9 targeted T-DNA inserts integrated into their genomic DNA. Since alfalfa is a tetraploid having potentially four allelic variations for a given gene, specific in-depth sequencing analysis is both needed and being conducted to determine the extent of CRISPR/Cas9-targeted gene DNA modification that has occurred in each allele for the ADT gene targeted. Sequencing of polymerase chain reaction (PCR) amplicons indicated mutations in targeted sites of one or all four alleles for the specific gene. Transgenic CRISPR/Cas9 alfalfa plants were bagged for self-pollination to fix the desirable traits. Seeds from transgenic T0 plants were plated onto selective medium to obtain T1 generation plants with fixed traits of interest for further analysis and determination of extent of inheritance of targeted gene traits. T1 plants are currently growing in the greenhouse and stems have been harvested for additional lignin analysis. Flowers are being bagged for the next round of self-pollination to enhance the fixation of the desired traits in the T2 generation. 1.3.2. Lignin analyses of alfalfa plants Lignin estimates in APEX system: Lignin estimates of individual alfalfa plant lines were performed. Estimated lignin levels of the low lignin alfalfa plants were lower as compared to the normal (WT) lignin lines grown. Lignin estimates of CRISPR/Cas9 transgenic alfalfa: Lignin estimate analyses were preliminarily performed using WT and transgenic alfalfa greenhouse grown. Estimated lignin levels of transgenic alfalfa plants were slightly lower than WT lines. 2. Future Work The next phase of our research is to sequentially complete the SVT and Experimental Verification Test experiments at NASA Kennedy Space Center (KSC), in preparation for approval and completion of our proposed ISS investigation. 3. Bibliography a. Corea, O.R., Ki, C., Cardenas, C.L., Kim, S.J., Brewer, S.E., Patten, A.M., Davin, L.B., and Lewis, N.G. (2012) Arogenate dehydratase isoenzymes profoundly and differentially modulate carbon flux into lignins. J. Biol. Chem. 287: 11446-11459. http://dx.doi.org/ b. Hixson, K.K., Marques, J.V., Wendler, J.P., McDermott, J.E., Weitz, K.K., Clauss, T.R., Monroe, M.E., Moore, R.J., Brown, J., Lipton, M.S., Bell, C.J., Pasa Tolic, L., Davin, L.B., and Lewis, N.G. (2021) New insights into lignification via network and multi-omics analyses of arogenate dehydratase knock-out mutants in Arabidopsis thaliana. Front. Plant Sci. 12: 664250. http://dx.doi.org/
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