Fiscal Year:	FY 2021	**Task Last Updated:**	FY 03/02/2021
PI Name:	Lewis, Norman G Ph.D.		
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		
PI Email:	lewisn@wsu.edu	**Fax:**	FY 509-335-8206
PI Organization Type:	UNIVERSITY	**Phone:**	509-335-2682
Organization Name:	Washington State University		
PI Address 1:	Institute of Biological Chemistry		
PI Address 2:	299 Clark Hall		
PI Web Page:	http://ibc.wsu.edu/		
City:	Pullman	**State:**	WA
Zip Code:	99164-6340	**Congressional District:**	5
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:**	12/31/2022
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:**	
No. of Bachelor's Candidates:	1	**Monitoring Center:**	NASA KSC
Contact Monitor:	Mickens, Matthew	**Contact Phone:**	
Contact Email:	Matthew.A.Mickens@nasa.gov		
Flight Program:			
Flight Assignment:			
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 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 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 ISS and Earth. Tissues (leaf, stem, and root) from ISS and Earth control will be collected after ca. 6-8 weeks growth, frozen (-160°C). They will be subjected to transcriptomic, proteomic, 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 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.
	Two Medicago species were considered for the ISS study, namely Medicago sativa (alfalfa) and Medicago truncatula (barrel medic). Alfalfa is a common forage and is well studied. However, it is also an outcrossing tetraploid that has largely been displaced as a model legume by barrel medic. The latter is a self-fertilizing diploid. Various experiments have been carried out to date to evaluate suitability of both species for ISS. Our focus has been on both hardware and manipulations needed for the proposed ISS study, i.e., in order to grow plants to a growth/developmental stage where we can convincingly demonstrate and evaluate successful nitrogen (N)-fixation and lignification in the microgravity environment. For the work described below, we have productive monthly teleconferences with Kennedy Space Center personnel to discuss our ongoing experimental strategy and results obtained. Progress made to date is as follows: Wicks: Key to our proposed experiments on ISS is the use of wicks, which both hold the seeds of Medicago species during launch and also provide an effective means of enabling watering the plants for both germination and growth/development. The wicks also have a crucial role for all of our downstream experiments, including for facile removal of plant shoots and roots from the plant growth containers. This is because the wicks allow for not only ready removal of root tissue, but also enable this to occur with the intact nodules in place. The wick experimental design can allow for both separation of nodules from the support medium and also for them to be collected quickly and efficiently. Hardware: Another experimental design variable is potential hardware for ISS using Veggie. We are currently assessing three different types of plant growth hardware, all certified and provided by NASA. These are the PILLOWS, PONDS, and APEX hardware, respectively. [Ed. Note: For more information on PILLOWS, PONDS, and APEX, see links referenced below.] All three have advantages and disadvantages. Our experiments are additionally designed to identify exactly what conditions and arrangements are best suited for our experiments to best succeed on the ISS. Using these three different forms of hardware, we have been testing and evaluating both plant species for their suitability to germinate and grow/develop in each, including establishing effects of light levels on growth/development, on nodulation, and on effects of ethylene with ethylene insensitive M. truncatula mutants. Additional work is being done on

Task Progress:	generating lignin-reduced Medicago lines, and on metabolomics analyses as described below. Germination: Alfalfa is generally easier to germinate than M. truncatula. Here, we routinely achieved good germination rates with alfalfa under conditions that mimic the wicking arrangement that we wish to deploy. However, we have not had reasonable success in getting high germination of M. truncatula under these same conditions. A second limitation we had was the APEX hardware system. While good germination occurs, the plants did not grow further. This appeared to be due to some incompatibility between Medicago and the Oasis foam material, and unrelated to the symbiosis. Light levels: We know from previous experience that alfalfa tolerates a variety of light conditions (it can be grown in continuous light), whereas M. truncatula is more sensitive to day length. We have tested both plant species under several LED (light emitting diode) lighting conditions, and identified conditions where both species grow well. By experimenting with both LED light intensity and color balance, we identified potentially optimal light conditions for growing our plants under the constraints of the VEGGIE plant growth hardware. Nodulation: This provided excellent results. Both Medicago species nodulated well, under some of the growth conditions tested, including in various ISS hardware configurations. Magenta boxes and ethylene: Our team has routinely grown alfalfa in closed magenta boxes, whereas M. truncatula does not grow well. We investigated whether this difference might be due to the known greater sensitivity of M. truncatula to ethylene, a plant hormone, by testing the growth of an ethylene insensitive mutant of M. truncatula in closed boxes. The mutant M. truncatula still grew very poorly in closed boxes, indicating that it is not suitable for these conditions. We are uncertain what the explanation is but do not think alfalfa presents a similar challenge. Lignin reduced alfalfa: An additional aspect of our experimental design is to use low lignin Medicago lines for our study on ISS. This work is progressing smoothly. The purpose here is in developing capabilities to be able to more readily biodegrade and recycle non-usable plant material, i.e., tissues which could not be used for consumption (in this case, tissues other than the usable part for forage). To do this, we need lignin-reduced lines that are more suitable for future long-duration spaceflight missions and/or for extraterrestrial colonization, i.e., where C/N recycling is optimal. Our overall strategy is to evaluate the level of C and N recovery attainable from such modified tissues via biodegradation, versus wild type lines. We have a dual-pronged approach to obtain lignin-reduced plants. The first is to down-regulate arogenate dehydratases as previously carried out with Arabidopsis thaliana. Here we are employing CRISPR/Cas9 genome editing, as this technology allows for creation of multiple mutants in a single step, thus time-consuming crosses and/or backcrosses are not needed. We are en route to generate single and multiple gene knockouts in alfalfa by targeting a single ADT gene with two or four guide RNAs, and/or by targeting two ADT genes with specific guide RNAs. For each gene (MsADT4 and MsADT5), sgRNA was designed (Synthego CRISPR Design Tool) to mitigate the potential for low-off target potential. M. sativa variety “LADAK” plants have been grown in sterile tissue culture containers in MS agar medium under 16 h of 120 mm fluorescent light conditions at 22°C. Total RNA was then prepared from 4-week-old leaves from these plants using a Sigma Spectrum Plant Total RNA Kit protocol. An Invitrogen SuperScript III First Strand Synthesis System was next used to prepare cDNA from 2 µg of the total RNA. The cDNA is currently being used in RT-PCR experiments to isolate the arogenate dehydratase gene homologs using primers designed from M. truncatula homologs that were previously identified using NCBI BLAST procedures. Once these M. sativa homologs are identified and sequenced completely, they will be subjected to analysis using the CRISPR Design Tool to identify 20 bp target DNA regions within exons for the purpose of knocking out the specific gene-of-interest. These sgRNA target regions will next be cloned into a binary vector containing the Cas9 protein and transformed into Agrobacterium tumefaciens for use in obtaining transgenic CRISPR/Cas9 lignin-reduced Medicago plants. Metabolomic analyses of M. truncatula and alfalfa species: Initially, two 5-6 week old plants each of M. truncatula and alfalfa plant lines were individually harvested. For metabolite extraction and analyses, each individual plant (containing leaves, stems, and roots) were collected, flash frozen in liquid nitrogen, and kept at -80°C. Next, each tissue type of M. truncatula and alfalfa plant lines was individually ground to a fine powder with liquid nitrogen using a mortar and pestle. A known amount (150-200 mg × 3) of each frozen pulverized tissue sample was weighed into a 2 ml Eppendorf tube and flash frozen in liquid nitrogen, to which ice-cold MeOH:H2O (1:1) containing 0.1 M naringenin as an internal standard was individually added. Samples were then individually sonicated for 10 minutes in ice-cold water, vortexed for few seconds, and centrifuged for 1 min at 4°C (16,000g). To the contents, ice-cold chloroform was next added, sonicated for 10 minutes in ice-cold water, vortexed for few seconds, and centrifuged for 15 min at 4°C (16,000g). Each aqueous methanol extract was individually separated and subjected to UPLC-qTOF-MS for metabolite analyses using Waters Acquity Ultra Performance LC system (Waters) equipped with a photodiode array (PDA) detector (Waters) coupled to a XevoTM G2 QTof mass spectrometer (Waters MS Technologies, Manchester, UK). Each chloroform extract and pellet were then separated and stored at –80°C for further analysis. From the LC-MS analysis, identification of metabolites in M. truncatula and alfalfa plant tissue types were performed by comparing their masses with reported literature data. Some ~100 metabolites (with putative identification, mainly belonging to flavonoid and saponin natural product classes), were individually annotated from leaves, stem and roots of each Medicago species. In sum, the alfalfa lines seem to offer the most promise for the proposed ISS studies, but we are still evaluating the hardware configuration. Links for Further Reference PILLOWS rooting packet: https:// PONDS/Passive Orbital Nutrient Delivery System: https:// APEX/Advanced Plant Experiment: https://
Bibliography Type:	Description: (Last Updated: 01/22/2025)