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Project Title:	Dissecting Beneficial Plant-Microbe Interactions and Their Efficacy in the ISS Spaceflight Environment, a Model Study		
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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, 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 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. Evaluation of Passive Orbital Nutrient Delivery System (PONDS) and APEX (Advanced Plant Experiment) Systems 1.1. Establishing N-fixing symbiosis on the ISS Responsibilities here included trying to anticipate and solve problems that might arise in translating ground-based procedures used to set up N-fixing symbiosis with Medicago species to formats used on the ISS. We have made considerable progress and are nearing the point where plausible procedures are contemplated for the ISS. Major issues faced were choice and validation of plants and symbionts, use of NASA certified growth platforms, and establishing routine methods for plant growth and periodic monitoring. 1.2. Choice of plants and symbionts We originally identified Medicago sativa and M. truncatula as candidate plant species, together with their respective bacterial symbionts, Sinorhizobium meliloti and S. medicae. M. truncatula is a diploid model for alfalfa, a major forage crop, and there were significant reasons for its consideration. However, M. truncatula grew poorly in containers and was more sensitive to irregularities in light. We therefore chose alfalfa, since it is generally more robust, and established appropriate growing conditions with its Ladak cultivar, which is routinely grown in the western part of the United States. Ladak forms a good symbiosis with S. medicae Rm1021, and there is considerable literature describing the bacterial symbiosis with various alfalfa cultivars. We also have recently shown that Rm1021 forms a satisfactory symbiosis with a low-lignin alfalfa strain, and a related one with normal levels of alfalfa lignin. 1.3. Optimizing plant growth hardware and conditions for conducting an effective alfalfa-Sinorhizobium symbiosis study on ISS LED lighting and experimental conditions: We established a small facility for testing various LED lighting conditions to determine optimal light intensity and color ratio for alfalfa growth. As carbon dioxide (CO_2) levels on the ISS average 3,500 ppm with relative humidity (RH) levels consistently at 45-50%, we tested both conditions in combination with previously optimized lighting levels determined at “normal” CO_2 and lower ambient humidity to evaluate alfalfa growth. After 6 weeks, alfalfa plants grown in 3500 ppm CO_2/45% RH grew significantly taller, with larger diameter, denser stems than those of plants grown in a 410 ppm CO_2 and 30% RH. These results indicate that the environment on board the ISS is suitable for growing alfalfa, at least with respect to humidity and CO_2.

Task Progress:

APEX plant growth chambers: NASA-supplied APEX growth containers were evaluated for the ability to grow alfalfa in symbiosis with S. meliloti. Experiments with the APEX configuration gave good growth of alfalfa, containing a developed root system with N-fixing nodules.

PONDS plant growth hardware: NASA-supplied PONDS plant growth containers were tested for the ability to grow alfalfa in symbiosis with S. meliloti Rm 1021 under previously optimized growing conditions. PONDS plant containers produced healthy plants after 6 weeks of growth, and contained N-fixing nodules of normal size, shape, and color.

Inoculation techniques: In typical ground-based symbiosis experiments, two-day old seedlings were hand inoculated with Rhizobium symbiont. However, ISS experiments require that inoculum be included in pre-packaged plant growth hardware. We tested several different inoculation strategies, all of which were successful. These included: lyophilized Rhizobium powder applied to seeds or wicks; Rhizobium infused guar gum glue; seeds coated with Rhizobium paste; and wicks soaked in bacterial cultures then dried. More experiments will determine which technique will be selected for the ISS experiment, but the current data indicate a very high probability of succeeding.

2. Lignin-Reduced Alfalfa

We have taken 2 approaches to obtain lignin-reduced alfalfa lines. The first lignin-reduced alfalfa line was obtained as seed, and the second deploys CRISPR/Cas9 to generate lignin-reduced alfalfa.

2.1. Low lignin alfalfa

Low lignin alfalfa is being grown from seed to assess their growth/development and lignin-reduction characteristics in the configurations above, with comparison to another alfalfa line of ‘normal’ lignin content.

2.2. Genetic engineering for lignin-reduced alfalfa

2.2.1. Cloning

Previous studies in our lab involving downregulation of arogenate dehydratase (ADT) genes in Arabidopsis thaliana resulted in reduced lignin plants (1,2). [Ed. Note: footnotes correspond to References at the end of the Task Progress section.] Therefore, we searched the National Center for Biotechnology Information (NCBI) database for homologs to these ADT genes in Medicago, since the latter was to be used for our currently planned ISS experiments. Initially, we obtained ADT homologs from Medicago truncatula and then later isolated the corresponding genes from cDNA preparations of M. sativa.

M. sativa variety “Ladak” plants were grown in sterile tissue culture containers in Murashige and Skoog (MS) agar medium. Total RNA and then DNA were prepared from 4-week-old leaves, this cDNA being used in RT-PCR experiments to isolate ADT homologs with primers designed from M. truncatula ADT.

CRISPR/Cas9 gene-editing was selected to disable ADT genes, potentially allowing for multiple gene knock-out targets in a single effort. Sequences were submitted to the Medicago datasets in CRISPRdirect and other plant CRISPR databases to detect and select gRNAs to target specific sequences for CRISPR/Cas9 genome editing with minimal off-targeting.

Methods developed by the Voytas lab (University of Minnesota) were used to clone multiple targets into a single T-DNA binary vector containing Csy4 binding sites and gRNA repeat regions, driven by the CmYLCV promoter (3). [Ed. Note: see References at end of Task Progress section.] The vector also contains the Cas9 enzyme driven by the 35S promoter, and final assembled vector constructs were sequenced to confirm that all components of the reaction were present. Confirmed vectors were transformed into Agrobacterium tumefaciens and verified by additional polymerase chain reaction (PCR) screening and resequencing.

Callus formation/plant regeneration/selection: Ladak cultivar plants were germinated and maintained in sterile conditions in Magenta box containers to provide leaf material for co-cultivation with Agrobacterium. Co-cultivated plant leaves were placed on medium containing hormones to induce callus development, with ticarcillin antibiotic to eliminate Agrobacterium after co-cultivation; neomycin phosphotransferase (npt) plant selectable marker was used for kanamycin resistance. Generated calli were then transferred to medium for shoot initiation, followed by plantlet formation. Plantlets were transferred to rooting medium and transferred to the greenhouse after rooting was sufficiently developed.

Currently, we are screening regenerated transgenic plants to detect mutations in targeted genes. Plants are being checked by PCR analysis to confirm presence of the npt gene selectable marker, the T-DNA insert, and specific ADT3 or ADT6 genes to use for subcloning for sequence analysis.

2.2.2. Transgenic plant screening approaches to detect CRISPR/Cas9 mutations

The tetraploid nature of alfalfa requires additional methods to confirm mutated targeted regions in the ADT genes. This includes restriction digestion of PCR amplicons, and primer sets for annealing to targeted regions to detect mutations.

Transgenic plant PCR amplified products are currently being cloned into a pCR4-TOPO vector to screen individual colonies for sequencing to detect variations in all four alleles of the tetraploid alfalfa. These results will be used to confirm allelic mutations arising in the ADT genes targeted in transgenic plants.

2.3. Metabolomic and lignin analyses of alfalfa lines

Metabolomic analyses for different tissues of wild type (WT) alfalfa plant lines, grown under two different light regimens, were carried out. Initially, ~3 to 4 plants of 5 week old alfalfa plants grown under 18- and 24-hour light schedules were selected. Alfalfa plants grown under the 24-hour lighting had ~1.1 × higher plant biomass compared to those grown under 18-hour lighting.

Metabolite extraction and analyses of leaf, stem, root, and nodule tissues from each plant were performed. The aqueous methanol extracts of individual tissue samples, obtained after initially pulverizing them with liquid nitrogen and later sonicating with MeOH: H2O, were subjected to UPLC-qTOF-MS for metabolite analyses.

Following liquid chromatography–mass spectrometry (LC-MS) analysis, identification of metabolites in each alfalfa plant was performed after normalization of metadata to the internal standard naringenin, followed by comparing their masses with reported literature data. The major metabolites (putative identification) were flavones in leaves (~44), stems (~35), roots (~30), and nodules (~25), respectively.

To determine if there was any significant variation in relative metabolite levels identified in each alfalfa tissue sample grown under 18- and 24-hour lighting, a statistical analysis -- i.e., orthogonal partial least squares discriminant analysis (OPLSDA) and heatmaps -- using web server MetaboAnalyst 5.0 was performed. OPLSDA plots of each individual tissue sample showed a clear segregation of metabolite clusters for plants grown under 18- and 24-hour light regimens under the growth conditions employed. Next, lignin estimates were performed to determine their levels in alfalfa plants grown under 18- and 24-hour lighting regimens. Pulverized individual stem samples were subjected to organic solvent extraction, followed by a water wash, then freeze-dried and later subjected to thioacidolysis by treating with dioxane-ethanethiol in presence of boron trifluoride etherate at 100 °C. Monomeric thioacidolysis derived products released from each sample were quantified, with estimated lignin levels of the alfalfa plants grown under 24-hour lighting having very slightly lower content (may not be statistically significant) as compared to both 18-hour lighting and greenhouse growth conditions.

References

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