Microgravity-Science and Technology (2018) 30:491–502
https://doi.org/10.1007/s12217-018-9619-4

ORIGINAL ARTICLE

The Impact of Simulated Microgravity on the Growth of Different Genotypes of the Model Legume Plant *Medicago truncatula*

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Received: 15 December 2017 / Accepted: 30 April 2018 / Published online: 8 May 2018

Abstract

Simulated microgravity has been a useful tool to help understand plant development in altered gravity conditions. Thirty-one genotypes of the legume plant *Medicago truncatula* were grown in either simulated microgravity on a rotating clinostat, or in a static, vertical environment. Twenty morphological features were measured and compared between these two gravity treatments. Within-species genotypic variation was a significant predictor of the phenotypic response to gravity treatment in 100% of the measured morphological and growth features. In addition, there was a genotype–environment interaction (G × E) for 45% of the response variables, including shoot relative growth rate ($p < 0.0005$), median number of roots ($p \sim 0.02$), and root dry mass ($p < 0.005$). Our studies demonstrate that genotype does play a significant role in *M. truncatula* morphology and affects the response of plants to the gravity treatment, influencing both the magnitude and direction of the gravity response. These findings are discussed in the context of improving future studies in plant space biology by controlling for genotypic differences. Thus, manipulation of genotype effects, in combination with *M. truncatula*'s symbiotic relationships with bacteria and fungi, will be important for optimizing legumes for cultivation on long-term space missions.

Keywords Clinorotation · Gravitropism · Legumes · *Medicago truncatula* · Simulated microgravity · Space biology

Introduction

While the history of human space flight has focused primarily on the development of research facilities located in Lower Earth Orbit (LEO), such as Skylab, Salyut, Mir, and most recently the International Space Station (ISS) (Sherwood 2011), there has been a shift by the National Aeronautics Space Agency (NASA) and international space agencies to push farther afield – aiming at near-Earth asteroids, the Moon, and Mars (Ansdell et al. 2011). Food, oxygen, water, and waste disposal needs for LEO research facilities have, thus far, been met through resupply missions from Earth, which have been both costly and a circumscribing factor to the practical radius of human space flight (Ferl et al. 2002).

Extended-duration missions at farther distances will require a self-sustaining Advanced Life Support (ALS) system to recycle waste and provide for the nutritional needs of the crew (National Research Council 2015). Many approaches to utilizing ALS have been proposed, although plant-based solutions are an obvious primary contender, due to the natural and inherent properties of oxygen and food production of plants, as well as their carbon dioxide and gray water recycling (Monje et al. 2003; Lehto et al. 2006; Kiss et al. 2014; Vandenbrink and Kiss 2016). There are several difficulties with crop-farming in space, including the amount of physical space required for traditional cultivation.

True microgravity can only be experienced either in space, such as via orbiting platforms including the ISS, or for shorter durations in Ground Based Facilities (GBF) such as drop towers, sounding rockets, or parabolic flights (Herranz et al. 2013; Kiss 2015; Pletser et al. 2017). One of the major considerations when using GBFs for biological research is that they lack an accounting of space radiation (Ferl et al. 2002; Wolverton and Kiss 2009; Vandenbrink and Kiss 2016). There may be instances where this is of benefit, as radiation could be a confounding variable for the microgravity response.

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Flying an experiment on the ISS is extraordinarily costly (Ansdell et al. 2011; Kiss 2015), although commercial and academic endeavors over the last decade have made significant advances in the pursuit of more affordable conduits for space research, particularly with regard to the development of miniaturized satellites (CubeSats) (Ansdell et al. 2011; Babuscia et al. 2015; Ciaralli et al. 2015, 2016; Scholz and Juang 2015). There are also several privately-funded American spaceflight services companies, such as Blue Origin and SpaceX, that are working to develop and refine truly reusable rockets, to make space research much more accessible. However, all of these methods are expensive, and troubleshooting on Earth is a prudent and critical step prior to flying experiments (National Research Council 2015).

Table 1 Accession designations of *M. truncatula* in Mt 4.0 SNP GWAS dataset (*Medicago* Hapmap ID)

HM001[a]	HM044[b]	HM082	HM130	HM177	HM217	HM270	*HM103*[b]
HM002[a]	HM045	HM083	HM131	HM178	HM218	HM271	*HM140*[b]
HM003[a]	HM046[a]	HM084	HM133	HM179	HM219	HM276	*HM204*[b]
HM004	HM047[b]	HM085	HM134	HM180	HM220	HM277	*HM255*[b]
HM005[a]	HM048[b]	HM086	HM135	HM181	HM221	HM278	*HM257*[b]
HM006[a]	HM049[b]	HM087	HM138[b]	HM182[a]	HM222	HM279	*HM258*[b]
HM007[a]	HM050[b]	HM088	HM139	HM1183[a]	HM223	HM280	*HM263*[b]
HM008[a]	HM051[b]	HM089	HM141	HM184[b]	HM224	HM287	*HM264*[b]
HM009[a]	HM052[a]	HM091[b]	HM143	HM185	HM225	HM288[b]	*HM272*[b]
HM010[a]	HM053	HM092	HM145[b]	HM186	HM226	HM289	*HM274*[b]
HM011[b]	HM054	HM093	HM146[b]	HM187	HM227	HM290[b]	*HM318*[b]
HM012[b]	HM055[b]	HM095	HM147	HM188	HM228	HM293[b]	*HM324*[b]
HM013[b]	HM056[a]	HM096	HM148	HM189	HM229	HM294	*HM325*[b]
HM014[a]	HM057[b]	HM097	HM149	HM190	HM230	HM295[b]	*HM326*[b]
HM015[a]	HM058[a]	HM098	HM150[b]	HM191	HM231	HM296[b]	*HM330*[b]
HM016[a]	HM059	HM099	HM151	HM192	HM232	HM297[b]	*HM331*[b]
HM019[b]	HM060[a]	HM101	HM152[a]	HM193	HM233	HM298[b]	*HM333*[b]
HM020-l	HM061[b]	HM105[a]	HM153	HM194	HM234	HM299[b]	*HM334*[b]
HM021	HM062	HM106	HM154[b]	HM195	HM235	HM300	*HM337*[b]
HM023	HM063	HM107	HM155	HM196	HM236	HM301[b]	*HM338*[b]
HM024	HM064	HM108[b]	HM156	HM197[b]	HM237	HM302[b]	
HM025	HM065[a]	HM109	HM157	HM198	HM238	HM304	
HM026[b]	HM066[b]	HM111	HM159	HM199	HM239	HM305[b]	
HM027[a]	HM067	HM112	HM160	HM200	HM240	HM306[b]	
HM028[a]	HM068	HM114	HM161	HM201[b]	HM241	HM307	
HM031	HM069	HM115	HM162	HM202	HM242	HM308[b]	
HM032[a]	HM070	HM117	HM163	HM203[b]	HM243	HM309[b]	
HM033[a]	HM071[b]	HM118	HM164	HM205	HM244	HM310	
HM034[a]	HM072	HM119	HM165	HM206	HM245	HM311[b]	
HM035[a]	HM073	HM120	HM166	HM207	HM253	HM312	
HM036[a]	HM074	HM121	HM167	HM208	HM256	HM313	
HM037[b]	HM075	HM122	HM168	HM209	HM259[b]	HM314	
HM038[b]	HM076	HM124	HM169	HM210	HM260[b]	HM315	
HM039[a]	HM077	HM125	HM170[b]	HM211	HM262	HM316	
HM040[a]	HM078	HM126	HM172	HM212	HM266[b]		
HM041[b]	HM079	HM127	HM173	HM213	HM267[b]		
HM042[a]	HM080	HM128	HM175	HM214	HM268[b]		
HM043[b]	HM081	HM129	HM176	HM215	HM269[b]		

In italics: HMID accessions from outside the Mt 4.0 SNP GWAS dataset that were also included in this study

[a]Accessions with at least one duplicate for both gravity treatments

[b]Accessions which were successfully cultivated to full term, with at least one duplicate

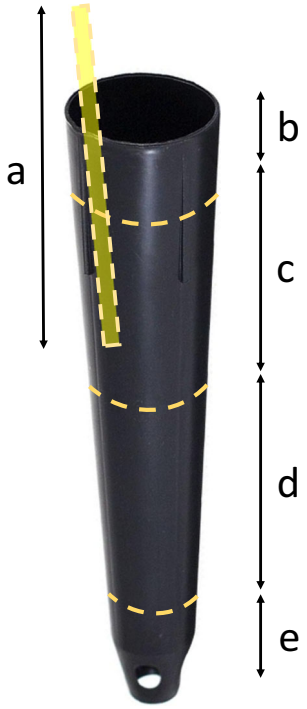

Fig. 1 Diagram of the containers (termed "cone-tainers") that were used to grow the seedlings of *Medicago truncatula* in these studies. The different regions of the containers contain: a) 6.35 mm diameter plastic tubing for watering; b) Foam plug; c) Sand; d) Sand:pebbles in a 2:1 ratio; and e) Cotton wool. These cone-tainers with seedlings were placed onto a rotating clinostat

Clinorotation, which is the horizontal rotation of plants about an axis such that the gravity vector is constantly changing, is another GBF that has become a common tool for simulating microgravity in plants to develop and refine experiments prior to actual spaceflight (Kraft et al. 2000; Herranz et al. 2013; Brungs et al. 2016). Clinorotation can be an effective proxy for microgravity for many parameters, as the primary known plant gravitropic mechanism is the perception of settling starch-dense vesicles (statoliths) by columella cell membranes in root tips (Kiss 2000; Kraft et al. 2000; Nakajima et al. 2017). In a constantly rotating environment the statoliths are continuously "falling" inside of the cell, never settling at the bottom as it is ever changing (Herranz et al. 2013). Several studies have found that clinorotation at 1 rpm can be an effective simulation of microgravity for plant life, depending on the parameters considered (Kraft et al. 2000; Hou et al. 2003; Herranz et al. 2013; Dauzart et al. 2016).

Plants in the Fabaceae family, commonly known as legumes, are an agriculturally, nutritionally, and economically valuable group of crops that include peas, soy, alfalfa, lentils, peanuts, and many beans (Graham 2003; Massa and Mitchell 2012; Wang et al. 2012; Varshney and Kudapa 2013). Their high nutritional value makes them a good candidate for cultivation on long-term space missions (Song et al. 2017).

While not directly used as food for humans, the plant *Medicago truncatula* nonetheless is an excellent model species for the Fabaceae family as it is a diminutive, fast-growing legume with a relatively small (~ 500 MBP) diploid genome. In addition, this plant readily forms symbiotic relationships (Miransari 2010; Dauzart et al. 2016) with both bacteria (nodules) and fungi (mycorrhizae). Since *M. truncatula* is a model plant system, there are a number of large scale genetic projects regarding this organism. For example, the *Medicago* Hapmap Project (http://www.medicagohapmap.org/) is a collaboration among the University of Minnesota, the National Center for Genome Resources (NCGR), Boyce Thompson Institute (BTI), J. Craig Venter Institute (JCVI), Hamline University, INRA-Montpellier, ENSAT-Toulouse,

Table 2 Hoagland's nutrient solution used in our studies. A concentrated stock solution was made for each component and sterilized via autoclaving [except for $Ca(NO_3)_2$ which was sterile filtered]

Component	Individual stock solution concentration	Volume of stock solution aliquot to add to 1 L to make 1X Hoagland's nutrient solution
KNO_3	2 M	2.5 mL
$Ca(NO_3)_2$	0.5 M	10 mL
EDTA-Fe^{+2}	0.04 M	1.5 mL
$MgSO_4$	2 M	1 mL
KH_2PO_4 (pH to 6.0)	1 M	0.5 mL
H_3BO_3	46 μM	1 mL
$MnCl_2$	9.1 μM	1 mL
$ZnSO_4$	0.7 μM	1 mL
$CuSO_4$	2 μM	1 mL
Na_2MoO_4	0.5 μM	1 mL
1M NH_4NO_3	1 M	1 mL

From these stocks, an aliquot was added to a container and brought to volume at 1L with water to make a 1X Hoagland's nutrient solution. This 1X solution was diluted further (1/8 strength) for use in our experiments

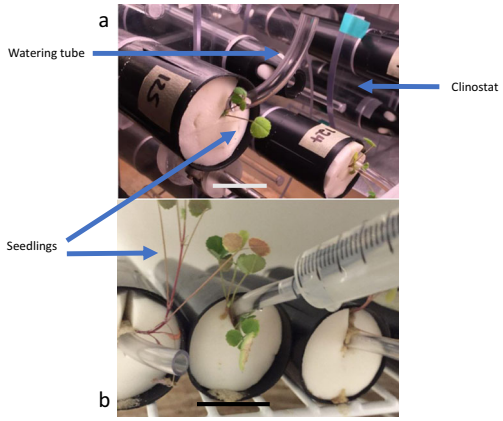

Fig. 2 *Medicago truncatula* seedlings growing in cone-tainers, sealed with foam plugs and watering tubes. **a** Seedlings rotating on the clinostat; **b** Seedlings being watered in cone-tainers via a syringe. Scale bars = 3 cm

and the Noble Foundation. "Hapmap" refers to haplotype mapping, or the mapping of genomic segments with shared ancestry. This consortium has sequenced 384 inbred lines of *Medicago* – predominantly *M. truncatula*, using Illumina Next-Generation Sequencing (NGS) technology and published the data online. As a component to their project, they have made available true-breeding seeds for each of these lines. Their goal is to create a free, accessible, genome-wide association (GWA) mapping resource for the plant research community.

GWA studies (GWAS) are observational studies in which genetic variance between individuals is analyzed to see if it is associated with a phenotypic trait. The germplasm made available by the *Medicago* Hapmap Project is all true-breeding, minimizing heterozygosity. This allows for the collection of high-resolution single nucleotide polymorphisms (SNPs), insertions/ deletions (INDELs) and copy number variants (CNVs). These data can be compiled and used as a basis for haplotype identification, as well as a novel way to look at population structure.

The present study was designed to characterize the morphological plasticity among *M. truncatula* genotypes and to investigate whether the growth of each genotype responds to simulated microgravity in a similar manner and with the same magnitude. The *Medicago* Hapmap project involves mapping haplotype associations in symbiosis-related phenotypes between *Medicago* individuals, with a view to uncovering genotype:phenotype associations related to symbiotic success. Similarly, we intend to create a GWA mapping framework with the goal of uncovering genotype:phenotype associations related to microgravity success.

We hypothesized that, overall, clinorotated plants would exhibit phenotypic differences, specifically in terms of growth parameters, compared to plants that developed vertically at $1g$. This hypothesis is supported by a large body of research, and would also be a confirmation of our own findings (Miyamoto et al. 1999; Kraft et al. 2000; Aarrouf

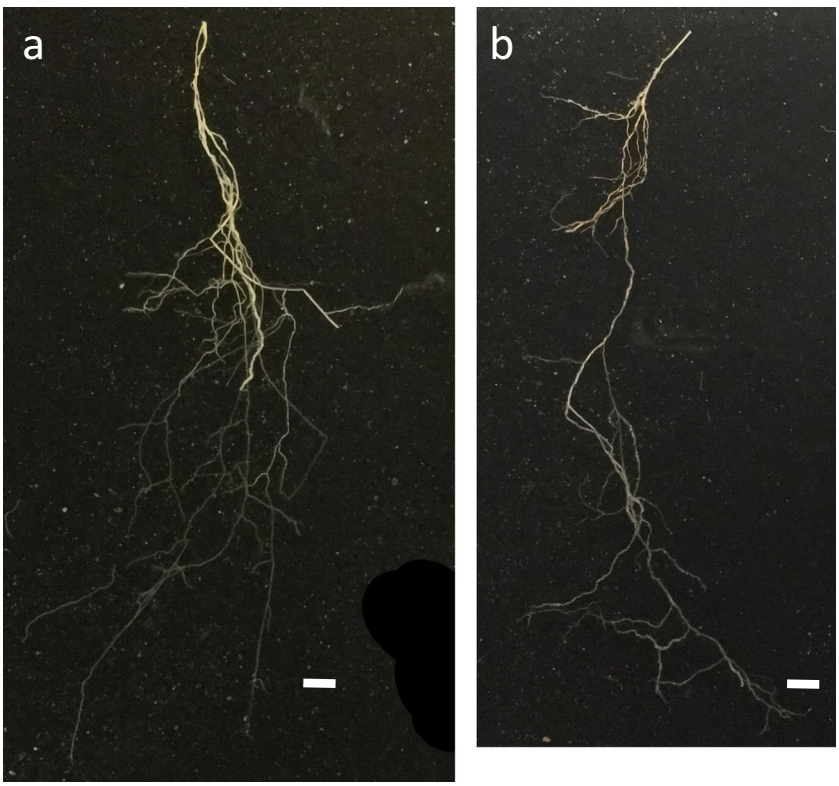

Fig. 3 Images of seedlings at harvest (36-days old). **a** Clinorotated HM001 ready for image analysis; **b** Vertically grown HM001 ready for image analysis. Note the differences in the pattern of root growth and root architecture in the HM001 strain growing in these different gravity conditions. Scale bars = 1 cm

et al. 2003; Hou et al. 2003; Kern et al. 2005; Hoshino et al. 2007; Herranz et al. 2013; Soh et al. 2015; Dauzart et al. 2016). Furthermore, we hypothesized that genotypic variation correlates with plastic and varied responses to gravity. This concept is important because, in the long term, we want to study more closely how various combinations of symbioses and genotypes will affect the gravity response (Dauzart et al. 2016). The *absence* of genotype-specific morphological variation in response to different gravity conditions would not eliminate the possibility of interaction effects manifesting when symbioses are considered.

Previous research has shown the limitations of using only a few genotypes in most space biology studies (Kiss et al. 2000; Vandenbrink and Kiss 2016), and the broader scope of this work is to reassess how we perform plant space biology experiments, and how much we can extrapolate from results gleaned from only one or two genotypes. Thus, in this report, we studied whether genotypes within the *Medicago truncatula* species behave differently from one another under clinorotation, and if some variants are more plastic than others, exhibiting a differential response to gravity.

Materials and Methods

Plant Material and Genotype Selection

We studied *Medicago truncatula*, which is considered a model legume system. Genotype selection was based primarily on the 262 *M. truncatula* accessions from the Mt 4.0 SNP GWAS data set, which was the latest available from the *Medicago* Hapmap project at the start of our experiments. (Table 1; http://www.medicagohapmap.org/downloads/mt40). Each accession is designated an alias beginning with "HM" and followed by a 3-digit number. Twenty-six accessions (HM001 - HM016, HM019, HM021, HM023 - HM028, and HM101) had been sequenced to 15X average aligned depth. The remaining accessions were

Table 3 Definitions of all the growth and development parameters used in these studies

Response variable	Definition
Root length (mm)	Length from farthest root tip to origin of primary root
Shoot length (mm)	Length of aerial tissue from origin to tip
Root fresh biomass (g)	Mass of the roots taken immediately at harvest. Dabbed dry with a Kim wipe, then weighed
Shoot fresh biomass (g)	Mass of the aerial tissue taken immediately at harvest. Dabbed dry with a Kim wipe, then weighed
Root dry mass (g)	Mass of the roots after tissue had been desiccated
Shoot dry mass (g)	Desiccated aerial tissue mass
Total fresh biomass (g)	Sum of Root Fresh Biomass and Shoot Fresh Biomass
RGR (g/g/day)	Relative growth rate from the time seedling was transplanted on its gravity treatment, to the time of its harvest (based on fresh mass of plant)
Shoot RGR (mm/mm/day)	Relative growth rate of only the aerial shoot tissue from the time seedling was transplanted on its gravity treatment, to the time of its harvest (based on length of shoots)
Root RGR (mm/mm/day)	Relative growth rate of only the root tissue from the time seedling was transplanted on its gravity treatment, to the time of its harvest (based on length of roots)
SRL (mm/g)	Specific Root Length: the ratio of root length to dry root mass
Average root width (mm)	The mean value of the root width estimation computed for all pixels of the medial axis of the entire root system
Network bushiness	The ratio of the maximum to the median number of roots
Maximum number of roots	After sorting the number of roots crossing a horizontal line from smallest to largest, the maximum number is considered to be the 84th-percentile value (one standard deviation)
Median number of roots	Result of a vertical line sweep in which the number of roots that crossed a horizontal line was estimated, and then the median of all values for the extent of the network was calculated
Network area (mm^2)	Number of network pixels in the image of the root system
Network perimeter (mm)	Total number of pixels connected to a background pixel (using an 8-nearest neighbor neighborhood)
Network surface area (mm^2)	The sum of the local surface area at each pixel of the network skeleton, as approximated by a tubular shape whose radius was estimated from the image
Network length (mm)	Total number of pixels in the network skeleton
Network volume (mm^3)	Sum of the local volume at each pixel of the network skeleton, as approximated by a tubular shape whose radius was estimated from the image

sequenced to an average aligned depth of ~6X (Branca et al. 2011; Stanton-Geddes et al. 2013). This deep sequencing coverage (aligned to the reference genome) allows for the identification of single-nucleotide polymorphisms (SNPs) and other genetic sequence differences between accessions. In addition, some germplasm from outside of this dataset was cultivated, due to availability and cultivation success (Table 1). In general, germination rates were low (~40%), and mortality during the first 10 days was high (~60%). However, once seedlings reached 15-d old, they were extremely robust, and very few individuals were lost for the remainder of the experiment.

Germination of Seeds

Seeds of each genotype were scarified using > 99.7% (v/v) sulfuric acid for 15 min, vortexed briefly, then rinsed four times with deionized (DI) water using 5 inversions between each decanting. Seeds were then surface-sterilized in 30% (v/v) bleach for 10 min and again rinsed 4X in DI water. Surface-sterilized seeds were placed in ~7 mL fresh DI water and shaken at 1000 rpm for 4 h, then germinated in upside down, sterile, Parafilm-sealed 10-cm Petri dishes for 36 h. Seedlings were sown into containers termed "cone-tainers" (Stuewe and Sons, Oregon, USA) plugged with cotton wool, and half filled with an autoclaved sand:pebble (in a 2:1 mixture), saturated with DI water, and topped with autoclaved sand (Fig. 1). Sown seedlings were sprayed 10 times with a 1/8 strength Hoagland's nutrient solution (Table 2) then grown under a 16:8 light-dark cycle at 20–22 °C with a fluence rate of ~150 μmol·m^{-2}·s^{-1} from "cool white" fluorescent lamps. Seedlings were watered as needed for 15 d via a spray bottle of 1/8 strength Hoagland's nutrient solution. The *Medicago truncatula* Handbook (Barker et al. 2006; Garcia et al. 2006) was used in the development of these procedures.

Transplanting of Seedlings

At day 15, the healthy seedlings were removed from the cone-tainers for initial growth parameter measurements and transplanting. During transplanting, seedlings were rinsed in DI water, photographed, measured for root and shoot length, weighed, then transplanted back into cone-tainers

Table 4 P-values from 2-way ANOVAs used to analyze the growth and development data

Growth parameters (response variables)	*p*-values		
	Genotype	Gravity	Interaction
Root length (mm)	1.97E-05****	2.03E-02**	2.05E-01*
Shoot length (mm)	6.27E-41****	1.52E-07****	1.38E-05****
Root fresh biomass (ln(g))	1.07E-41****	8.43E-02*	8.13E-01*
Shoot fresh biomass (g)	7.98E-34****	6.67E-07****	1.77E-01*
Root dry mass (g)	5.15E-10****	1.01E-02**	6.20E-01*
Shoot dry mass (g)	6.13E-35****	3.82E-03***	4.05E-03***
Total fresh biomass (g)	2.37E-49****	8.17E-01*	7.08E-01*
RGR (g/g/day)	4.44E-33****	4.82E-01*	3.22E-01*
Shoot RGR (mm/mm/day)	1.38E-36****	2.27E-06****	1.96E-05****
Root RGR (mm/mm/day)	4.18E-26****	8.27E-01*	1.22E-01*
SRL (mm/g)	4.52E-14****	3.03E-02**	4.69E-01*
Average root width (mm)	1.68E-14****	2.51E-01*	5.54E-02*
Network bushiness	3.17E-08****	6.67E-02*	6.49E-02*
Maximum number of roots	1.06E-12****	6.02E-03**	4.18E-02**
Median number of roots	1.84E-14****	6.74E-03**	2.09E-02**
Network area (mm^2)	1.05E-09****	1.32E-03***	4.46E-02**
Network perimeter (mm)	8.36E-11****	1.96E-03***	3.57E-02**
Network surface area (mm^2)	5.13E-10****	1.57E-03***	4.67E-02**
Network length (mm)	2.47E-11****	2.53E-03***	3.88E-02**
Network volume (mm^3)	2.52E-09****	1.93E-03***	7.93E-02*

P-values denote degree of correlation between the response variable and either the genotype or gravity treatment, with the final column showing p-values for interaction effects between genotype and gravity for each growth parameter. Asterisks refers to degree of statistical significance, with a *p*-value threshold of 0.05
$*p > 0.05$; $**p < 0.05$; $***p < 0.005$; $****p < 0.0005$

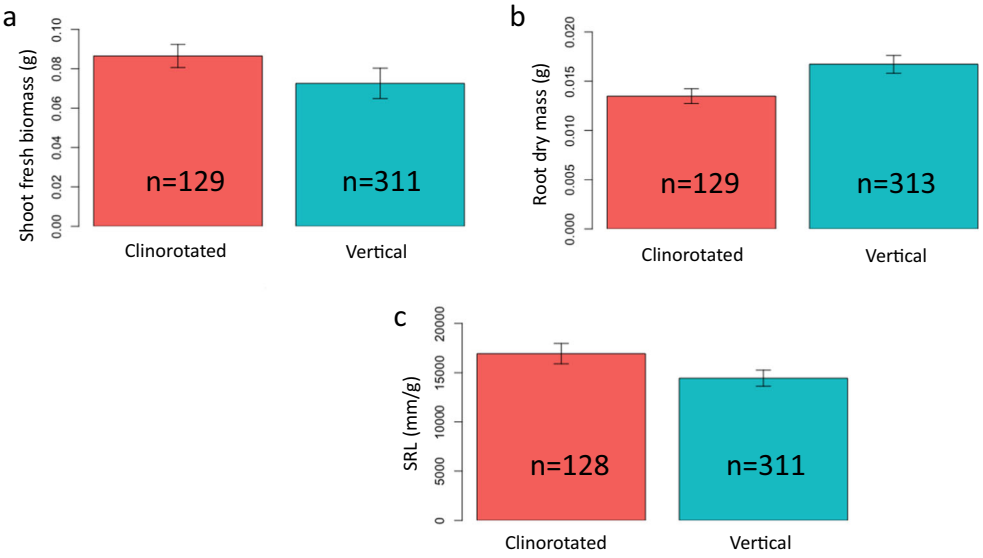

Fig. 4 The effects of gravity treatment (x-axis) on growth parameters (y-axis) of the entire population of *Medicago truncatula* examined. Error bars signify +/− 1 SE. **a** Biomass of fresh shoots; **b** Biomass of dried roots; **c** SRL (Specific Root Length). Sample size for each experiment is given directly on the figures. Statistically significant differential responses to simulated microgravity were seen in terms the fresh biomass of shoots (a; $p < 0.0005$), the dry mass of roots (b; $p \sim 0.01$), and the Specific Root Length (c; $p \sim 0.03$)

and watered thoroughly with DI water. A 9-cm long plastic tube (6.35 mm diameter) was planted with them, covered with a thin layer of dry sand, then secured in place by wrapping the shoot and tubing with a foam plug (Fly Plugs 89140-960, VWR) (Fig. 1). At this point, each plant was transferred to its gravity treatment – either returned to a vertical stand, or placed horizontally onto a ~1 rpm clinostat (Dauzart et al. 2016) (Fig. 2). Clinostats rotated continuously for the duration of the experiment. All plants were watered via a 10-mL syringe through their tube every other day with either 8 mL DI water, or alternately, 8 mL of 1/8 strength Hoagland's nutrient solution (Fig. 2).

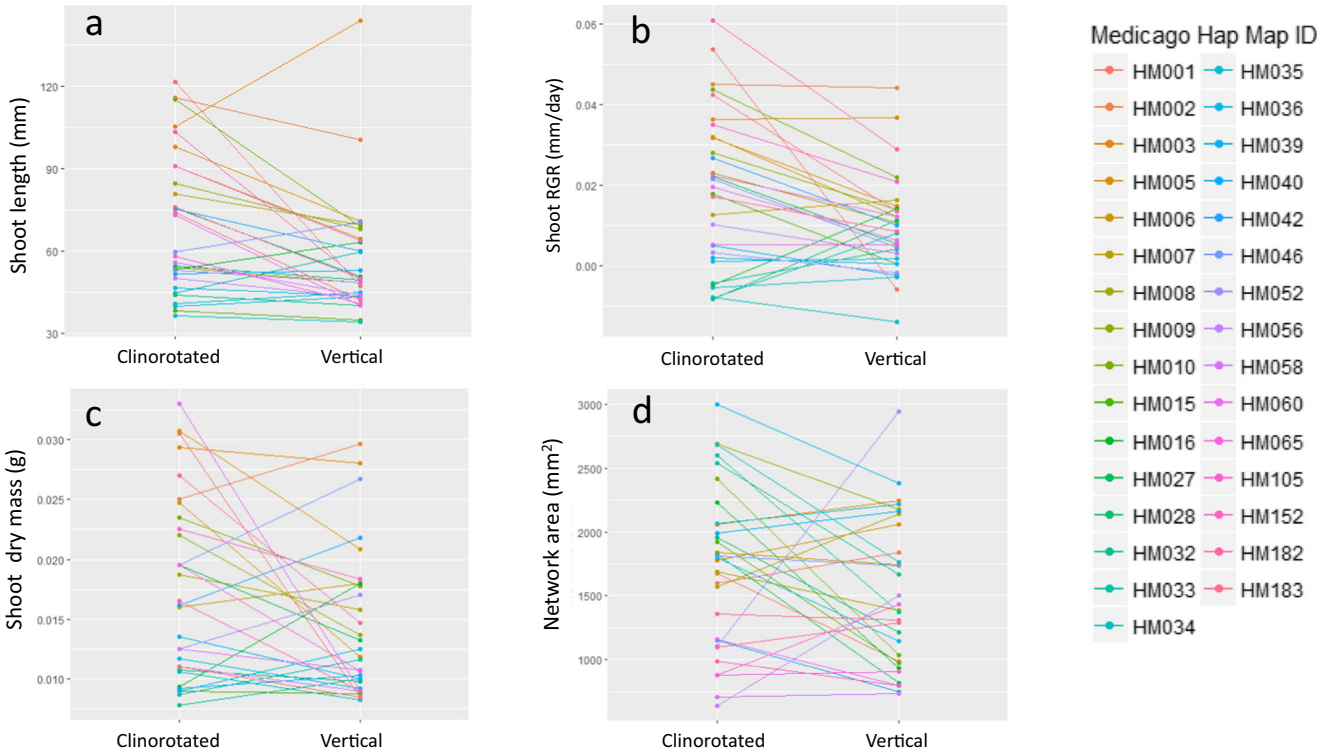

Fig. 5 Interaction effects of gravity treatment (x-axis) with genotypes on various growth/developmental parameters (y-axis) of *Medicago truncatula.* Some strains showed wide variations in response to the gravity treatment while other strains showed little difference between the two gravity treatments. Replicates for each gravity treatment ranged from 2–6 individuals

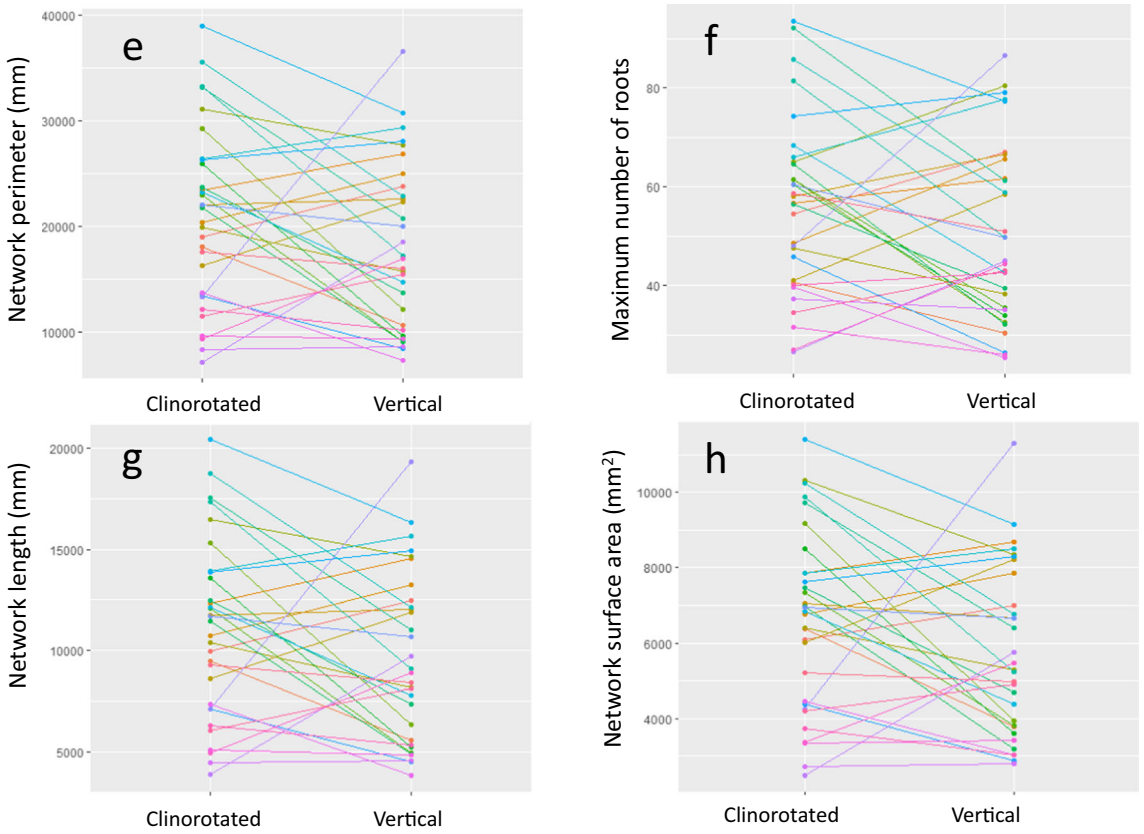

Fig. 5 (continued)

Harvest and Data Collection

Twenty-one days after transplanting (36-days post germination), the plants were removed from their cone-tainers, rinsed with DI water, and photographed. They were each measured for root and shoot growth, as well as total fresh biomass accumulation. Image analysis was performed using the publicly available program GiA Roots (Galkovskyi et al. 2012) on all root network images. All images were taken from a fixed distance, and included a 10-mm reference for scale. After assessing accuracy of scale in seedling images, they were cropped or edited to remove the scale, as well as any visible shoots and any obvious background noise (Fig. 3), allowing GiA Roots to assess only the root network. Outputs from image analyzes were in units of pixels. Using the average conversion factor collected from ten images at random, all pixel units were converted to mm. Parameters measured and calculated are noted in Table 3. Relative growth rates (RGRs) are measurements of growth rate relative to size, and they are sometimes termed the exponential or continuous growth rate. RGRs were calculated using the logarithmic equation $\mathrm{RGR} = (\ln \mathrm{W2} - \ln \mathrm{W1})/(\mathrm{t2} - \mathrm{t1})$, with t1 the date of transplant, and t2 the harvest/data collection date. W denotes the measurement of growth recorded at either time point (t1 or t2). Shoot and root RGRs are based on length, and the simple RGR response is based on mass.

Two-way ANOVAs were performed for all measured morphological response variables listed and defined in Table 3, using gravity treatment (vertically grown or clinorotated), genotype (HapMap ID or HMID), and the gravity x genotype interaction. The p-values for these tests are shown in Table 4. All calculations and statistical analyzes were computed using R (R Core Team 2016). Any data point for which there was not at least one duplicate (from both the same genotype and the same gravity treatment) was discarded, so that all the data points used for statistical analyzes were averages across replicates. The number of individuals that passed this threshold was $n = 451$.

Results

In the first part of our study, we considered the effects of gravity on growth parameters across the entire population of *M. truncatula* (for these experiments, not considering genotype; Fig. 3). Statistically significant and clear differential responses to simulated microgravity were seen in terms

of the following parameters: the fresh biomass of shoots (Fig. 4a; $p < 0.0005$), the dry mass of roots (Fig. 4b; $p \sim 0.01$), and the Specific Root Length (SRL) (Fig. 4c; $p \sim 0.03$) (Table 4). Differences in gravity response also occurred in plant root length ($p \sim 0.02$), but the standard error of each gravity group overlapped.

However, much more prevalent than a clear main gravity response for the entire population was the presence of interaction effects, i.e., the effect of gravity on an individual's growth morphology depended on genotype (genotype–environment interaction or GxE). These interaction effects are illustrated in reaction norm graphs in Fig. 5, which show how, for some genotypes, the effect of gravity was to increase the response phenotype, while for others there was a clear decrease. This significant GxE interaction was observed in 9 of the 20 phenotypes analyzed (45%). The strongest interaction effects were seen in some of the shoot-related phenotypes. Shoot length and shoot RGR were both extremely affected ($p < 0.0005$; Fig. 5a, b), and shoot dry mass was also affected strongly by the interaction between gravity and genotype (Fig. 5c; $p < 0.005$). Other significant interaction effects between genotype and gravity treatment played a role in several of the root phenotypes, including network area, network perimeter, maximum number of roots, and network length, all with $p \sim 0.04$ (Fig. 5d, e, f, g), as well as network surface area (Fig. 5h; $p < 0.05$), and median number of roots ($p \sim 0.02$).

Genotype affected all measured response variables (Table 3). Figure 6 illustrates some examples of morphological traits that exhibited *only* this main effect. These examples showed no differential responses to gravity treatment when considered a whole, but there were strong correlations between growth and genotype. Several root-related phenotypes were in this category, including root RGR and root fresh biomass (Fig. 6a, b; $p < 0.0005$), along with network bushiness ($p < 0.0005$) and average root width ($p < 0.0005$). Additionally, both RGR based on mass (Fig. 6c), and the total fresh biomass, showed a morphological response to genotype alone ($p < 0.0005$) and not to the gravity treatment.

Taken together, these results show that not only does genotype play a significant role in *M. truncatula* morphology, it

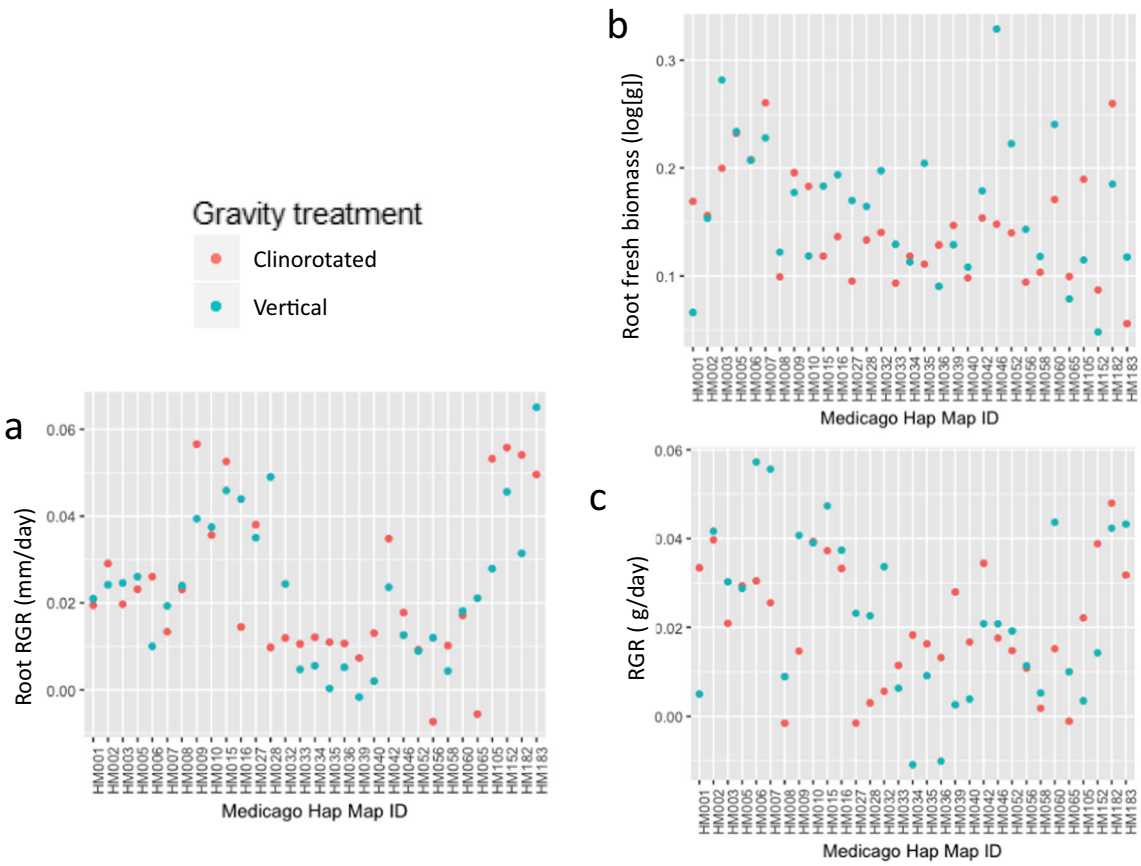

Fig. 6 Phenotypic responses that were not significantly affected by gravity treatment but were affected by genotypic variation. While gravity did not significantly affect Root RGR, Root Fresh Biomass or RGR, genotypic variation was shown to explain a significant amount of phenotypic variation among the individuals. Replicates for each gravity treatment ranged from 2–6 individuals

frequently affects the plant's response to gravity treatment, influencing *both* the magnitude and direction of the gravity response.

Discussion

Based on the present studies, we can make two overarching conclusions. First, that genotypic variation in *M. truncatula* significantly affected all measured response variables (Table 3). Second, that within-species genotype variation caused a plastic (G × E) interaction with the gravity treatment, making the phenotypic response to simulated microgravity differ among genotypes. Furthermore, these results suggest that we must be cautious in our interpretations of plant experiments dealing with gravitropism and microgravity that do not take genotype into account (Kiss et al. 2000; Vandenbrink and Kiss 2016).

A vast literature of plant space biology research has been published and considered in a number of review articles (Ferl et al. 2002; Wolverton and Kiss 2009; Kiss 2013). We must begin to assess how those findings can be extrapolated and utilized for future scientific inquiry and space exploration. Thus, it is critical that we acknowledge the limitations of our research and fill in any gaps. For example, when we perform reduced gravity experiments on *Arabidopsis thaliana*, what exactly does that mean? There are many genotypes of *A. thaliana* – over 1100 ecotypes sequenced already (http://1001genomes.org/), and researchers are already working on how to interpret and use these data (Gan et al. 2011).

While a few spaceflight studies consider genotype in their experimental design (Kiss et al. 2000), most do not. There are inherent difficulties involved in ameliorating these past approaches. For example, it would be untenable to use hundreds or thousands of genetic variants within a species for all space or even ground-based experiments. A more practical approach to addressing this issue would be to gather data from a large foundation of ground-based experiments delineating which loci are correlated with different phenotypic outcomes, within and across species, and use those data along with NGS analyses of the variants being tested as a lens through which to interpret subsequent results. GWAS seeks to reconnect traits back to their underlying genetics (Korte and Farlow 2013), and the more we elucidate those connections, the better we can statistically control for them in future spaceflight studies.

We acknowledge the scale of this task. Beyond aligning morphological outcomes with SNP, INDEL, and CNV data, any serious model used to assess future plant space biology data would also have to consider epigenetics and even epistatic effects (Duncan et al. 2014; Kooke et al. 2015). This prospect sounds daunting, but there may well be patterns and themes in these data. As plants have all evolved under an unchanging gravity vector since the origins of plant life on this planet, it seems reasonable to assume that they have not evolved specific plastic mechanisms for tolerating gravity stress. However, we know that plants do have these specific plastic mechanisms. Despite this characteristic, plants exhibit gravitropic responses to varying extents, and we know some of the underlying mechanisms involved (Kiss 2000; Vandenbrink and Kiss 2016). It could be that the loci strongly correlated with gravitropic responses are random, but it seems more likely that they will be linked in some fashion. This knowledge, along with the ever-increasing speed and accuracy of NGS platforms and bioinformatics as a whole, should enable us to account for plasticity in phenotypic responses across genotypes in the future. These types of data are the next step in precise and effective genetic engineering of plants for optimal vigor and productivity in future space travel (Ferl et al. 2002; Lehto et al. 2006).

Overall, *Medicago* genotypes grown under conditions of clinorotation experienced an increase in root and shoot biomass accumulation when compared to 1-*g* controls. However, this phenomena was genotype specific. The majority of genotypes tested showed an increase in shoot length when compared to 1-*g* controls (Fig. 5a), as well as biomass accumulation in the root networks (Fig. 5d–h). The relative root growth (mm/mm/day) was shown to be increased in clinorotated samples in 18 of the 31 genotypes, suggesting increases in root growth in conditions of simulated microgravity is highly genotype dependent (Fig. 6a). In previous experiments, root growth has been shown to increase during exposure to simulated microgravity (Hoson et al. 1997; Hilaire et al. 1996; Levine and Piastuch 2005). However, similar studies have also reported no change in root growth rate (Volkmann et al. 1986; Perbal et al. 1987) or that roots were shorter in conditions of true microgravity (Cowles et al. 1984; Iversen et al. 1996). The varying results from these previous studies are not surprising, as the results presented here indicate that root growth rates can differ even within distinct genotypes of a single species.

The increase in root growth rate associated with conditions of microgravity can also cause an increase in the cell proliferation rate of the plant. A study analyzing *Arabidopsis thaliana* growth rates in real and simulated microgravity showed an increase in the number of cells in the root meristem (Matia et al. 2010). Conversely, the size of cells in the meristem as well as the production of ribosomes (indicators of cell growth) appear to be reduced. This observation has been attributed to a shortening of the G2 cell growth phase, resulting in a larger number of smaller cells, and is correlated with an increase in the overall seedling root length.

It is important to note that the above (e.g. Matia et al. 2010) studies were conducted with a single *Arabidopsis* ecotype (Columbia). Thus, similar to the results observed in the present study, it is possible that the responses may differ depending on the genetic background of the genotype tested. Therefore, in determining which plants are suited for optimal growth in conditions of microgravity, or the reduced gravity experienced on the Moon or Mars, analysis of a wide range of plant genotypes should be conducted to find the strain best suited for growth in space (Vandenbrink and Kiss 2016).

Further studies are needed to expand and confirm our results. One possible future approach would be to use the *Medicago* Hapmap resources to perform a Genome-Wide Association Study (GWAS). This method would enable us to map trait loci and begin to understand how individual haplotypes correlate to phenotypic plasticity and responses to altered gravity states. These proposed studies would also have to be replicated in true microgravity, as clinorotation is a useful but limited microgravity simulator for plant experiments (Kraft et al. 2000; Herranz et al. 2013; Kiss 2015).

Of particular interest to space biology would be to then explore how *M. truncatula* symbioses with plant-growth-promoting rhizobia and arbuscular mycorrhizal fungi affect plant genotypes differently (Miransari 2010; Nadeem et al. 2014) with a view to better understand these connections and, ultimately, using these interactions to improve and refine crop cultivars and growing conditions for space explorations (Dauzart et al. 2016). Extended-duration space travel and human space missions beyond Lower Earth Orbit (LEO) will require reliable and sustainable Advanced Life Support (ALS) systems. Thus, manipulation of genotype, in combination with *M. truncatula*'s symbiotic relationships with rhizobacteria and arbuscular mycorrhizal fungi, will be important for optimizing legume productivity for cultivation on long-term space missions.

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