Mutualism Within a Simulated Microgravity Environment - *Piriformospora indica* Promotes the Growth of *Medicago truncatula*

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ABSTRACT

The endophytic fungus, *Piriformospora indica*, developed a subepidermal infection within *Medicago truncatula* at 1 g and at simulated microgravity over a period of 15 days, resulting in intracellular colonization of mature host tissue. At 1 g, *P. indica* inoculation affected the growth and morphology of *M. truncatula*, predominantly roots. Inoculated *M. truncatula* had a significantly greater number of roots (102%), total root length (88%), and dry root weight (25%) than non-inoculated plants. Effects on shoot morphology of *P. indica* inoculated *M. truncatula* included longer (31%) and heavier (30%) shoots, along with increased leaf surface area (98%). *P. indica* retained the ability to promote the growth of *M. truncatula* under simulated microgravity conditions upon two dimensional clinostatic rotation, significantly increasing root number by 51% and root length by 48%. These physiological and morphological changes may mitigate biotic and abiotic stresses that would otherwise limit crop productivity.

INTRODUCTION

Leguminous plants (beans, peas, alfalfa, vetch, etc.) are important to humans and natural and agricultural ecosystems, as they account for 27% of primary crop production and are cultivated on 12% to 15% of the Earth's arable soil (Graham and Vance, 2003). *M. truncatula* (Barrel medic) is extensively used as a model species of the legume family due to its relatively small genome size, ease of growth, and ability to induce transformations (Trinh et al., 1998). Legumes develop a symbiotic relationship with rhizobia bacteria, which enables the fixation of atmospheric nitrogen, replenishing depleted soils for future cultivations, and achieving sustainability under environmental conditions that would otherwise limit crop productivity (Graham and Vance, 2003).

Plant/microbe systems will play a major role in long-term bioregenerative life support systems on board spacecraft and long duration space missions by providing fresh food and recycling air, water, and waste products (Ferl et al., 2002). Establishing successful plant/microbe mutualisms would improve and increase plant growth, stress tolerance under microgravity, and increase flight crew independence by reducing re-supply costs during long-term missions. *P. indica*, a root endophytic fungus isolated from the soils of the Indian Thar Desert, Rajasthan (Varma et al., 1999; Verma et al., 1998), has been shown to induce host growth promotion, increase seed yield, and increase resistance to abiotic and biotic stresses (Bagde et al., 2011; Deshmukh et al., 2006; Peskan-Berghofer et al., 2004; Varma et al., 1999; Waller et al., 2005),

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thus displaying its potential as a plant growth-promoting and stress-alleviating bioinoculant (Sarma *et al.*, 2011). *P. indica* displays a lack of host-specificity by establishing symbiotic relationships with a wide range of plant hosts, including monocots and dicots (Deshmukh *et al.*, 2006; Peskan-Berghofer *et al.*, 2004; Varma *et al.*, 1999). This seemingly non-specific host recognition directly contrasts with the highly specific symbiosis between *Sinorhizobium meliloti* and specific members of Leguminosae (Jones *et al.*, 2007). The non-specific root colonization strategies of *P. indica* have been described for barley (Deshmukh *et al.*, 2006) and *Arabidopsis* (Peskan-Berghofer *et al.*, 2004). Both species display similar infection patterns, resulting in intracellular colonization of the host's mature cortical cells (Deshmukh *et al.*, 2006; Peskan-Berghofer *et al.*, 2004; Schäfer and Kogel, 2009). This intracellular root colonization is associated with host cell death (Deshmukh *et al.*, 2006; Jacobs *et al.*, 2011), which is intriguing as detrimental effects do not arise with infection.

Understanding the impacts of reduced-gravity environments on symbiotic plant/microbe associations is critical before inclusion in a functioning regenerative life support system. As plant growth and metabolism are altered in microgravity, leading to physiological changes which may affect host defenses, plants become more susceptible to infection by pathogens and organisms that normally do not cause disease (Bishop *et al.*, 1997; Tripathy *et al.*, 1996). The effect of the unique space environment on cell-to-cell communications and biochemical interaction between organisms – like those involved in symbiotic associations – can result in an alternative ecological balance that redefines the relationship between host and commensal, or between host and opportunistic pathogen (Stutte and Roberts, 2012). The ability of *P. indica* to override plant defense mechanisms during infection (Jacobs *et al.*, 2011) is thus of concern during microgravity simulation, as there is evidence that the virulence of plant pathogens increases under such conditions (Ryba-White *et al.*, 2001). A previous study by Bishop *et al.* (1997) showed that a non-pathogenic fungal species (*Neophodium*) can become pathogenic during spaceflight; however, a more recent study carried out on the well-defined mutualistic symbiosis between *M. truncatula* and root nodule forming

bacteria *S. meliloti* during spaceflight suggested that both organisms cultivated in microgravity still have the potential to establish a symbiotic interaction (Stutte and Roberts, 2012).

A better understanding of the limitations of these mutualistic associations and their adaption to this unique environment is required, since such investigations may also uncover insights into how microbial populations may be managed in terrestrial settings to enhance crop production. Clinorotation is used on the ground for investigations as an analog for microgravity on biological systems (Albrecht-Buehler, 1992). This vertical rotation through the gravity vector overwhelms the organisms with an orientational stress, similar to that perceived in microgravity (Albrecht-Buehler, 1992; Dedolph and Dipert, (1971).

The aims of the present investigation were: 1) to test the hypothesis that *M. truncatula* growth would increase through a mutualistic association with *P. indica*, 2) to establish the timeline for infection and colonization strategy within the *M. truncatula* roots, and 3) to test whether this mutualistic association develops under simulated microgravity conditions.

MATERIALS AND METHODS

M. truncatula Seed Scarification and Sterilization

Seeds of *M. truncatula* Gaertn cv. Jemelong A17 (gift from Carroll Vance, USDA-ARS, St. Paul, MN) were extracted from seed pods (typically 6-8 seeds/pod) and seed coats were gently, mechanically scarified on fine-grade (150 grit) sandpaper until there were visible signs of seed coat abrasion. Seeds were then sterilized in a 33% bleach solution for three minutes and rinsed with several washes of sterile H₂O (Garcia *et al.*, 2006).

P. indica Axenic Cultivation and Inoculum Preparation

P. indica was obtained from the DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) and initially grown from spores from freezer stocks on 60 mm Petri dishes containing MS media (Murashige and Skoog, 1962) within a 30°C incubator for 6 days. *P. indica* was then harvested by adding 8 mL of sterile, deionized (DI) H₂O to Petri dishes, and gently agitating and carefully removing hyphae and spores from the agar surface with a sterile plate spreader.

The solution was transferred to a 50 mL sterile centrifuge tube for use as an inoculant. A similar process was carried out on non-inoculated MS plates for use as treatment for non-inoculated control plants. A spore count via haemocytometer measured the spore concentration to be $3.3 \times 10^5/\text{mL}$. The spore solution was diluted with 3.3 mL of the control inoculum to generate a final working stock of 1.0×10^5 spores per mL as the fungal inoculant.

***M. truncatula* Seed Inoculation**

Sterile *M. truncatula* seeds were soaked in 15 μL of the fungal inoculum ($1.0 \times 10^5/\text{mL}$ or 1500 spores), or 15 μL of control treatment solution for five hours within the laminar flow hood for inoculated and non-inoculated plants, respectively.

Growth Conditions

All seeds were aseptically transferred to 10 mL of MS media, within individual sterile Petri dishes 15 mm x 60 mm (i.e., one seed per dish). The Petri dishes were sealed with Parafilm® and punctured with pin holes to allow additional gaseous exchange. The plates were then set up on a 2-D clinostat (described in next section) and transferred to a controlled environment chamber (CEC) (EGC model no. M48, Environmental Growth Chamber, Chagrin Falls,

Ohio) for 15 days. The CEC was set to the following parameters throughout experimentation: relative humidity 50%, temperature 22°C, CO₂ ambient, 16 hr light/8 hr dark photoperiod at 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetically active radiation (PAR), under GE F96T8/SPX41/HO fluorescent lamps.

Two-Dimensional (2-D) Clinostat Setup

Each individual Petri dish (containing one seed) was attached to a 15 cm (diameter) plastic disk. Eight Petri dishes in total (four control and four *P. indica* inoculated seeds) fit onto each disk and their formation lined the outer region of the disks (Figure 1). Four orientational treatments were prepared to complete the 2-D clinostat setup—vertical static (VS), vertical rotating (VR), horizontal static (HS), and horizontal rotating (HR) (Figure 1). Both of the rotating treatments were attached to an 8 rpm motor spinning at a centrifugal force of $5.3 \times 10^{-3}g$ throughout the entire experiment, whereas both static treatments remained fixed. The VR treatment simulates the microgravity stress due to the constant reorientation of cellular gravity perception as the disk spins (Perbal and Driss-Ecole, 2003). The other three treatments (VS, HS, HR) were set up as controls to account for the effect of centrifugal force/rotation and orientation on the interaction separately.

Figure 1. 2-D clinostat set up within the ECG, *M. truncatula* seeds are placed in the center of each Petri dish on MS media. Setup from a-d are horizontal static (HS), horizontal rotating (HR), vertical static (VS), and vertical rotating (VR), respectively. The VR (d) setup is an analog to simulate microgravity stress.

Biometric Image Analysis

All plant length measurements were determined from digital images using Image J (Abràmoff *et al.*, 2004) for image analysis. Scales were set using digital calibration (Abràmoff *et al.*, 2004).

P. indica Staining and Host Colonization Analysis Procedure

Over a period of days (day 4, 11, and 13) post-inoculation, individual control and treated samples were removed from CEC and their root systems were analyzed for *P. indica* infection. A simple ink-vinegar staining process was carried out (Pitet *et al.*, 2009) on the root samples, followed by analysis under a light microscope (Nikon Labophot). Briefly, the protocol required clearing the sample tissue of phenolics and unwanted pigments by heating in 10% KOH at 100°C and rinsing with water. The tissue was then fixed with 5% acetic acid, followed by additional rinsing with water. Each sample was then stained for 2 hours at room temperature in a 5% acetic acid/ 5% Schaeffer black ink solution. Lastly the ink/vinegar stain was decanted and the root tissue was de-stained by several washes with an acidified glycerol solution. The roots were then viewed under a light microscope and their images recorded with a handheld camera (Nikon Coolpix S3100).

Chlorophyll Pigment Extraction and Analysis

Total chlorophyll (a, b) and carotenoid concentrations per dry weight of sample were measured from dimethyl sulfoxide (DMSO) extracts (Lichtenthaler and Wellburn, 1983; Wellburn, 1994). Harvested shoot samples were frozen at -80°C and freeze dried. Samples pigments were then extracted in 4 mL DMSO and its UV-visible absorbance read from a Biotek Gen 5 plate reader over a spectra range of 300-700 nm and at individual spectra – 665, 649, and 480 nm. Chlorophyll a and b concentrations were then determined from three replicate mean absorbance values using the Wellburn equation described for DMSO extractions (Wellburn, 1994).

Statistical Analysis

The experiment was carried out with 4 repeats (4 un-inoculated and 4 *P. indica* inoculated) for each orientation, and was replicated four times. All of the collected data was combined together, giving a total of 16 un-

inoculated and 16 *P. indica* inoculated samples for each clinostatic setup described previously. Initially, to identify outliers, all data obtained was subjected to an interquartile outlier test using Excel (Microsoft Office Excel 2010), whereby a number of outliers were identified and removed from the data set. The remaining (post outlier removal) sample size (n) is displayed on respected growth characteristic figures. Therefore, based on the validity of the underlying assumptions (normality and homoscedasticity (both assessed using Minitab, Version 17)), the appropriate tool for statistical analysis was chosen (i.e., parametric, non-parametric tests) to measure the effect of *P. indica* inoculation on *M. truncatula* growth, clinorotation on *M. truncatula* growth, and interactions between *P. indica* inoculation and clinorotation. Data, which was found to meet the requirements for analysis of variance (ANOVA), was subjected to a one-way ANOVA with a critical difference of $P < 0.05$, whereas non-normal and data of unequal variance was subjected to the Kruskal-Wallis test, also with a critical difference of $P < 0.05$ using Minitab (Version 17).

RESULTS AND DISCUSSION

M. truncatula Root Colonization Strategy by *P. indica* Under 1 g and Simulated Microgravity Stress

To date, *P. indica* has not been reported to display distinct host-specificity, as it will colonize the root systems of many plant species, including monocots and dicots (Peskan-Berghofer *et al.*, 2004; Varma *et al.*, 1999; Waller *et al.*, 2005), both inter- and intracellularly, with asymptomatic results. The infection strategy by *P. indica* during host colonization has been described from its association with barley and *Arabidopsis* (Deshmukh *et al.*, 2006; Jacobs *et al.*, 2011; Peskan-Berghofer *et al.*, 2004; Schäfer and Kogel, 2009). In both species, *P. indica* initially colonizes root rhizodermal cells, followed by cortical tissue and intracellular colonization of cortex cell layers.

We report, to the best of our knowledge, the previously unrecorded infection strategy of *P. indica* within *M. truncatula*. *P. indica* and *M. truncatula* develop a mutualistic relationship, through fungal colonization of root tissue, which results in the intracellular establishment of *P. indica* within the maturation zone of the plant

root. *P. indica*'s propagation is not exclusive to host cells—it also grows externally on agar and root surface. This infection strategy is consistent with the infection strategies reported for barley (Deshmukh *et al.*, 2006) and *Arabidopsis* (Peskan-Berghofer *et al.*, 2004). The infection process is initiated by an extracellular establishment of *P. indica* along the topography of the outer epidermal layer of the root by days after inoculation (DAI) 4 (Figure 2A). By DAI

11, subepidermal fungal colonization is observed within the cortical tissue (Figure 2B), along with intracellular sporulation (Figure 2C). Figure 2C shows *P. indica* to have intracellularly colonized the more mature regions of the plant root tissue, completely occupying its host's cells with chlamydospores. By DAI 13, heavy fungal colonization/sporulation was observed within the maturation zone of *M. truncatula* roots (Figure 2D).

Figure 2. Infection strategy of *P. indica* within *M. truncatula* at 1 g. The infection was monitored with the use of an ink-vinegar stain, which adheres to chitin within *P. indica* appearing as blue. Root sections are marked as follows: epidermis (e), cortex (c), and vascular bundle (vb). (A) *P. indica* grows along the *M. truncatula* root topography during an initial extracellular establishment by DAI 4. (B) By DAI 11, subepidermal *P. indica* root colonization is witnessed within the inner cortical cells. (C) DAI 11, *P. indica* colonizes the mature cortex root tissue intracellularly, completely occupying its host cells with chlamydospores. (D) At DAI 13, heavy fungal colonization is seen within the maturation zone of the *M. truncatula* root. Scale bars on all images represent 50 μ m in length.

These findings are consistent with that reported from *P. indica*'s association with barley and *Arabidopsis* (Deshmukh *et al.*, 2006; Jacobs *et al.*, 2011), whereby *P. indica* sporulation increased within mature root tissue and was associated with the occurrence of host cell death. This suggests that *P. indica* either interferes with programmed cell death when forming a mutualism within its host, or actively senses the process and invades targeted dead

cells. This recently reported colonization strategy of the endophyte is intriguing, as it achieves this high volume of root cell death associated infection without causing detrimental effects to its host (Deshmukh *et al.*, 2006; Jacobs *et al.*, 2011). The same strategy is witnessed within *M. truncatula*, accompanied by an increase in *M. truncatula* growth (Figure 4).

Upon investigating whether or not *P. indica* was capable of infecting *M. truncatula* under

simulated microgravity, it was found that *P. indica* retained the ability to form an endosymbiotic association (Figure 3), with

intracellular sporulation being observed within mature host tissue by DAI 12.

Figure 3. Infection strategy of *P. indica* within *M. truncatula* at simulated microgravity. Root sections are marked as follows: epidermis (e), cortex (c), and vascular bundle (vb). (A) By DAI 12, subepidermal *P. indica* root colonization is witnessed within the inner cortical cells of *M. truncatula*. (B) Inner cortical cells of *M. truncatula* are intracellularly colonized by *P. indica* chlamydospores by DAI 12. Scale bars on all images represent 50 μm in length.

Very little literature exists relating to the microbial colonization of plants within microgravity environments. An experiment in the 1970s had a similar clinostatic setup, investigated the effects of gravity compensation on crown gall formation, and found that under simulated microgravity conditions the infection occurred (Kleinschuster *et al.*, 1975). Along with infection, the crown gall developed tumors that were said to be larger on gravity-compensated samples than those grown at 1 *g* (Kleinschuster *et al.*, 1975). Bishop *et al.* (1997) previously reported the presence of a strain of *Neotyphodium* (a known endophyte) within space-grown wheat. The fungal strain had colonized the wheat samples; however, in contrast to our observations, its colonization strategy could not be reported. *P. indica*'s colonization strategy involves an override of host defenses (Jacobs *et al.*, 2011) and is associated with host cell death (Deshmukh *et al.*, 2006), and is thus a concern during microgravity simulation (Ryba-White *et al.*, 2001). Interestingly, *P. indica* is closely associated with an endosymbiotic bacteria (*Rhizobium radiobacter*), which is known to also cause crown gall disease within hosts (Sharma *et al.*, 2008). This is a further concern under microgravity stress, as crown gall tumors are reported to enlarge under gravity compensation (Kleinschuster *et al.*, 1975). In this study,

however, colonized *M. truncatula* appeared healthy post-clinorotation.

Effect of *P. indica* Inoculation on *M. truncatula* Growth and Morphology at 1 *g*

The mechanism by which *P. indica* elicits its beneficial effect to its host is not well defined. Studies have linked various diffusible factors, such as extracellular phytohormone production by *P. indica* with the increase in plant growth (Sirrenberg *et al.*, 2007). Vadassery *et al.* (2008) found that *P. indica* produces low levels of auxin and high levels of cytokinin, and colonized *Arabidopsis* plants contained higher levels of cytokinin than uncolonized.

This study showed a positive effect of *P. indica* root colonization on the growth and morphology of *M. truncatula*. *M. truncatula* plants treated with *P. indica* appeared visibly healthy, while also displaying an increase in shoot and root length. Growth promotion in the aerial organs consisted of a 31% increase in total stem length (control 83 mm vs. treated 109 mm), 30% increase in shoot dry weight (control 15 mg vs. treated 20 mg), and 98% increase in total leaf surface area (control 126 mm² vs. treated 250 mm²) (Figure 4 D-F). The subterranean organs displayed the highest level of growth promotion, which consisted of a 102% increase in root number (control 23 vs. treated 47), 88% increase in total root length

(control 322 mm vs. treated 606 mm), and a 25% increase in dry root weight (control 7 mg vs. treated 9 mg) (Figure 4 A-C). This increased root growth promotion may enhance stress tolerance, such as within drought related areas where water is held deeper within the soil, as well as produce a greater yield of legume crop

for forage. Similar growth effects have been reported from *P. indica*'s association with micropropagated *Feronia limonia* (L.) Swingle (Vyas *et al.*, 2008). Also, the treatment with *P. indica* culture filtrate alone has been reported to induce and stimulate the same growth promoting response (Bagde *et al.*, 2011).

Figure 4. The effect of *P. indica* inoculation on *M. truncatula* growth characteristics after 15 days inoculation at 1 g (HS). All displayed data was found to be significant ($P < 0.05$), respective P values are included on each graph. Sample size is displayed as n.

The morphological differences witnessed in this investigation could be a result of hormone alterations, such as auxin (Davies, 2004). Exogenous auxin produced by rhizobacteria tends to promote root growth and branching

(Shahab *et al.*, 2009), and is consistent with Sirrenberg *et al.*, (2007) findings that auxin plays a role in *P. indica*'s growth-promoting effects. In contrast, Sirrenberg *et al.*, (2007) reported that auxin levels were not up-regulated

within treated *Arabidopsis* plants – while cytokinin levels were – and concluded that cytokinins were required for *P. indica*-induced cell division and growth promotion (Vadassery *et al.*, 2008). Another rhizospheric diffusible factor capable of eliciting such a growth-promoting response could be lipochitooligosaccharides (LCO) (Maillet *et al.*, 2011). Rhizobacteria and mycorrhizal fungi, which produce LCOs during host infection, have been shown to induce LCO-dependent lateral root formation (Maillet *et al.*, 2011; Olah *et al.*, 2005), similar to that induced by *P. indica*. LCO exudates alone are also capable of promoting the same induced lateral root formation response (Olah *et al.*, 2005), similar to *P. indica* fungal exudate (Bagde *et al.*, 2011). Interestingly enough, *P. indica* colonization is associated with an up-regulation of calcium levels within host tissue (Vadassery *et al.*, 2009), which happens to be essential for nodulation within *M. truncatula* and linked to the LCO signal transduction pathway (Olah *et al.*, 2005). However, to our knowledge, no investigation into the role of LCOs as a diffusible factor responsible for *P. indica*-induced growth promotion has been carried out.

The current investigation suggests that *M. truncatula*'s association with *P. indica* may also result in alterations to host physiology, as increased levels of host photosynthetic tissue were found with *P. indica* inoculation. Treated plants showed a 25% increase in total chlorophyll a and b levels, in comparison to non-treated control samples; however, this finding was not found to be significant (data not shown). Previous studies on *P. indica*'s effect on *Arabidopsis* under drought-stress also showed higher chlorophyll content and increased photosynthetic efficiency with *P. indica* inoculation (Sherameti *et al.*, 2008). Similar to arbuscular mycorrhizal fungi (AMF) symbiosis (Ceccarelli *et al.*, 2010; Yano-Melo *et al.*, 1999), such increases in leaf perimeter length, photosynthetic potential, and chlorophyll may result in increased carbon assimilation in *P. indica*-colonized plants, leading to faster development and higher biomass production.

Effect of Clinorotation on the Growth of *M. truncatula*

The effect of clinorotation on *M. truncatula* was investigated to determine if the rate of rotation and or the orientation of the 2-D set up had a significant effect on plant development, and ultimately if analog

microgravity conditions had an effect on the growth of *M. truncatula*. The HS (1 g) treatment was used as a control whereby, HR (1 g), VS (1 g), and VR (microgravity) were all used as comparisons. Analysis of *M. truncatula* plant growth on all four 2-D clinostatic setups yielded significant variations among HS and VS treatments (orientation change) at 1 g, and also between HS and VR treatments (from 1 g to microgravity) (Figure 5 A-F, HS vs. VS). Decreases in *M. truncatula* growth on the VS in comparison to the HS treatment (orientation change) may have been associated with a lower availability of nutrients to root zones, i.e., plants growing on the horizontal axis would fully penetrate into media, whereas those on the vertical axis may grow along the media. Nevertheless, the VR axis which simulates microgravity was found to be the strongest inhibitor of *M. truncatula* growth in relation to root growth, with significant differences recorded for root count (-42%, HS 36 vs. VR 25), root length (-53%, HS 458 mm vs. VR 299 mm), and dry root weight (-70%, HS 8 mg vs. VR 5 mg) (Figure 5 A-C, HS vs. VR). *M. truncatula* total stem length (-17%, HS 97 mm vs. VR 83 mm), shoot dry weight (-39%, HS 18 mg vs. VR 13 mg), and total leaf surface area (-46%, HS 180 mm² vs. VR 124 mm²) were also found to be strongly inhibited under analog microgravity conditions (Figure 5 D-F, HS vs. VR). The rate of rotation ($5.3 \times 10^{-3}g$) was found to not have a significant effect on *M. truncatula* development on the horizontal axis (1 g), as HS samples did not alter significantly to HR (Figure 5 A-F, HS vs. HR A-F). Taking all observations into account suggests that the orientational change from horizontal to vertical elicited an inhibitory effect on *M. truncatula* development, while rotation displayed a larger and significant inhibitory effect on the vertical axis only, showing a *M. truncatula* growth inhibitory effect under analog microgravity conditions.

Effect of *P. indica*'s Association with *M. truncatula* on the Morphological and Physiological Development of *M. truncatula* Under 2-D Clinorotation

Multiple studies have shown *P. indica*'s ability to mitigate biotic and abiotic stresses that would otherwise inhibit plant growth, with *P. indica* inoculation inducing salt tolerance, drought tolerance, pathogen

resistance, transfer shock tolerance, and resistance to low temperatures (Murphy *et al.*, 2014; Murugan, 2011; Sherameti *et al.*, 2008; Varma *et al.*, 1999; Waller *et al.*, 2005; Zarea

et al., 2012). To our knowledge, this is the first instance whereby *P. indica*'s association with a host plant grown under simulated microgravitational stress has been reported.

Figure 5. The effect of clinorotation on the development of *M. truncatula*. HR, VS, and VR (microgravity) growth measurements are all compared to HS (1 g). P values displayed above each column describe the level of significant difference between the respective columns and the HS column. P values < 0.05 were accepted as significant, whereas non-significance is depicted as NS. Sample size is represented as n.

Inoculation with *P. indica* had a significant effect on growth and morphology of *M. truncatula* under analog microgravity conditions. The most influential root count promotion was recorded on the HS (1 g)

treatment (102%), while the simulated microgravity (VR)-treated associates resulted in a 51% promotion (Figure 6 A). The same was found for total root length, with HS (1 g) displaying an 88% increase in growth, and

simulated microgravity (VR) samples displaying a lesser, but still substantial 48% increase in growth (Figure 6 B), all of which shows that analog microgravity conditions have a ‘nulling’ effect on *P. indica* induced *M. truncatula* root growth promotion. In addition, *P. indica* inoculated plants grown on the simulated microgravity setup showed similar growth stimulatory effects as that seen on the 1g plane, such as increased root weight, shoot weight, and total leaf surface area. These findings, however, were found to not be significant (data not shown).

Bishop *et al.* (1997) reported that a non-pathogenic strain of *Neotyphodium* (endophyte) became pathogenic under spaceflight, leading to detrimental effects on wheat growth. Here we report that although mitigated, *P. indica* still retains the ability to induce the root growth promotion of *M. truncatula* under analog microgravity conditions (Figure 6 A-B). This is an interesting observation as it suggests that gravity’s influence upon *M. truncatula* and or *P. indica* may play a role within the mutualistic association.

Figure 6. Root growth stimulatory effect of *P. indica* on the growth of *M. truncatula* at 1 g and simulated microgravity. P values shown represent the level of significant difference between non-inoculated and *P. indica* inoculated *M. truncatula* on HS (1 g) and VR (microgravity) growth conditions. Sample size is represented as n.

CONCLUSIONS

These findings demonstrate that the legume *M. truncatula* is colonized by the endophytic fungus *P. indica*, and that this infection results in the promotion of root and shoot growth of the host. The interaction represents an ideal combination to study the effect of *P. indica* on leguminous plants. The infection strategy is similar to that reported for barley and Arabidopsis, suggesting a common mode of infection between monocots and dicots. Colonization by *P. indica* was also observed under analog microgravity conditions, and the infection was similar to that of 1 g. *P. indica* infection also resulted in significant increase in growth of *M. truncatula* under analog microgravity conditions, albeit these were less than observed for 1 g. With regard to *P. indica*’s effect on host morphology, our observations suggest that *P. indica* LCOs (a previously un-associated diffusible factor) may

be contributing to root morphological alterations.

These results demonstrate that establishing plant/microbe symbiosis has potential to enhance the growth of plants under analog microgravity conditions, under mission-relative environmental conditions. Additional research is needed to understand how the analog microgravity environment mitigates the effect of *P. indica* on *M. truncatula* growth.

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