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Monitoring Bacterial Colonization and Maintenance on Arabidopsis thaliana Roots in a Floating Hydroponic System

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December 7, 2018

Dear Jaydev Upponi,

Enclosed please find our manuscript, "**Monitoring bacterial colonization and maintenance on the roots of *Arabidopsis thaliana* in a floating, hydroponic system**", which we are submitting to the Journal of Visual Education.

Rhizosphere colonization by plant growth-promoting bacteria (PGPB) can increase the health or development of host plants in the presence of diverse stressors compared to uncolonized plants, at least in laboratory conditions. However, when tested in field settings, treatments of plants with PGPBs often don't provide substantial benefits to crop growth. In an effort to understand this discrepancy between lab and field studies, we developed a hydroponic plant-growth assay to quantify species presence and visualize the spatial distribution of bacteria during initial colonization and following transfer into differing growth environments.

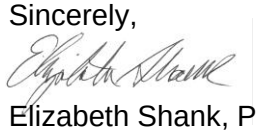
We validated our system's reproducibility and utility with the well-studied PGPB *Pseudomonas simiae*. We wanted a system that would be applicable to a range of bacteria species, and thus that had sufficient adaptability to account for the differing growth needs to a range of bacteria while also permitting exploration of field-like environmental stressors. Further, we wanted to support the health and development of the plant host. The hydroponic system we describe here allows bacteria to colonize in nutrients that are most conducive to their growth or in conditions of interest. We show that we can measure the presence of multiple bacterial species using this assay, which provides an alternative to sequencing-based studies of bacterial colonization of plant roots.

Future studies using this system may improve our understanding of how bacteria behave in multispecies plant microbiomes over time and in changing environmental conditions, with the potential to improve crop productivity.

The main manuscript has four figures and one supplemental figure.

We look forward to hearing from you.

Sincerely,



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TITLE:

Monitoring Bacterial Colonization and Maintenance on *Arabidopsis thaliana* Roots in a Floating Hydroponic System

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microbiology, microbial interactions, plant-microbe interactions, hydroponic, rhizosphere, plant biology, colonization, PGPR, PGPB, beneficial bacteria, bacterial community, microbiome

SUMMARY:

Here we describe a hydroponic plant growth assay to quantify species presence and visualize the spatial distribution of bacteria during initial colonization of plant roots and after their transfer into different growth environments.

ABSTRACT:

Bacteria form complex rhizosphere microbiomes shaped by interacting microbes, larger organisms, and the abiotic environment. Under laboratory conditions, rhizosphere colonization by plant growth-promoting bacteria (PGPB) can increase the health or the development of host plants relative to uncolonized plants. However, in field settings, bacterial treatments with PGPB often do not provide substantial benefits to crops. One explanation is that this may be due to loss of the PGPB during interactions with endogenous soil microbes over the lifespan of the plant. This possibility has been difficult to confirm, since most studies focus on the initial colonization rather than maintenance of PGPB within rhizosphere communities. It is hypothesized here that the assembly, coexistence, and maintenance of bacterial communities are shaped by deterministic features of the rhizosphere microenvironment, and that these interactions may impact PGPB survival in native settings. To study these behaviors, a hydroponic plant-growth assay is optimized using *Arabidopsis thaliana* to quantify and visualize the spatial distribution of bacteria during initial colonization of plant roots and after transfer to different growth environments. This system's reproducibility and utility are then validated with the well-studied PGPB *Pseudomonas simiae*. To investigate how the presence of multiple bacterial species may affect colonization and maintenance dynamics on the plant root, a model

community from three bacterial strains (an *Arthrobacter*, *Curtobacterium*, and *Microbacterium* species) originally isolated from the *A. thaliana* rhizosphere is constructed. It is shown that the presence of these diverse bacterial species can be measured using this hydroponic plant-maintanence assay, which provides an alternative to sequencing-based bacterial community studies. Future studies using this system may improve the understanding of bacterial behavior in multispecies plant microbiomes over time and in changing environmental conditions.

INTRODUCTION:

Crop destruction by bacterial and fungal diseases results in lowered food production and can severely disrupt global stability[1]. Based on the discovery that microbes in suppressive soils are responsible for increasing plant health[2], scientists have asked whether the plant microbiome can be leveraged to support plant growth by modifying the presence and abundance of particular bacterial species[3]. Bacteria found to aid in plant growth or development are collectively termed plant growth-promoting bacteria (PGPB). More recently, studies have shifted from simply identifying potential PGPB to understanding how interkingdom interactions in the soil, around roots, or in the rhizosphere (the area directly surrounding and including the root surface) may be impacting PGPB activity[4].

Rhizosphere colonization by PGPB can increase the health or the development of host plants in response to diverse stressors relative to uncolonized plants[5]. However, results are often more variable in native soil conditions compared to those observed in the closely controlled greenhouse and laboratory settings[6]. One hypothesis for this difference is that the growth or behavior of PGPB may be inhibited by native soil bacteria or fungi in the fields[7,8]. Beneficial effects by rhizosphere bacteria generally depend on the ability of the bacteria to 1) locate and move towards the root, 2) colonize the root through biofilm formation, and 3) interact with the host plant or pathogens via production of small molecule metabolites[7,9]. Any of these colonization behaviors may be affected by the presence and activity of neighboring microbes[10].

We designed a system to quantify and visualize these distinct bacterial colonization stages of the rhizosphere (**Figure 1**). This approach will facilitate studies investigating why long-term PGPB maintenance is sometimes not observed following transfer of plants into new environments, such as during the planting of pre-inoculated seedlings. *Arabidopsis thaliana* as were chosen as a plant model due to its extensive use in laboratory studies as well as the ample data available about its microbial interactions[11]. There are three stages in the system: 1) *A. thaliana* growth, 2) bacterial colonization, and 3) bacterial maintenance (see **Figure 1**). Because *A. thaliana* is a terrestrial plant, it was important to ensure that it was not suffering undue water stress in the hydroponic system[12]. Inspired by the methods used by Haney et al.[13], the seedlings are grown on plastic mesh to separate the shoot from the liquid growth medium. This system does not appear to compromise the health and development of the plant host, and it improves *A. thaliana* growth in liquid[11]. As the plant shoot floats above the surface, the roots are fully exposed to colonization by bacteria inoculated into the liquid bacterial growth medium. This permits bacteria of interest to be examined for colonization in nutrients that are most conducive to growth, while then shifting conditions to allow the plant to continue growing in a nutrient medium designed to support its growth. Both stages include steady shaking to

prevent anoxia of the root[13]. Bacteria can be visualized or quantified from the plant roots following transfer from either the colonization medium or the maintenance medium. This hydroponic system is very flexible, allowing experimental conditions and applied stresses to be easily altered depending on interests of the researchers.

This described method is important in the context of the larger body of literature about plant-microbe interactions because it provides a robust system for studying these interactions at the root surface while also being customizable to the growth preferences of different bacteria. Plant biology labs often perform plant-microbe colonization experiments on solid agar, allowing for only planar movement (if that) of bacteria while also requiring the potentially destructive manipulation of plants during subsequent transfer. In contrast, microbiology labs have frequently prioritized the health of the bacteria within their experiments, to the detriment of the plants[14,15]. These different priorities of plant- and microbiology-focused labs have historically made it difficult to compare results between these groups, since each typically optimizes experimental conditions to optimize their organism of interest[15]. The floating-mesh-plant-growth system described here prevents full plant submersion, a notable advantage to previous microbiology-oriented studies, while also temporarily optimizing the growth and survival of bacteria to facilitate colonization. Thus, the assay we present here may address concerns from both plant biologists (about over-hydration and tactile manipulation of the plant) while satisfying the criteria of microbiologists (allowing for different bacterial growth conditions and multiple species' interactions)[7]. This protocol is designed to be adaptable for use with various bacteria, plants, and environmental conditions.

PROTOCOL:

NOTE: The experimental setup is described for clarity and used to generate the representative results included in this report, but conditions can be modified as desired. All steps should be performed using PPE and following institutional and federal reccomendations for safety, according to the BSL status of the bacteria used.

1. Characterization of bacteria

1.1. Determine the morphology of bacteria on the growth medium agar plate. Resuspend cells at an approximate OD_{600} = 0.5 and plate a 1 µL volume onto agar medium of choice. Add X-gal to agar plates to a final concentration of 20 mg/mL to better differentiate individual members of the specific bacterial community. Grow at 24 °C or 30 °C until colonies form, then take pictures of and notes on colony morphology.

1.2. Define the correlation between each bacterial strain's optical density and the number of CFU (colony forming units) per mL[16]. Resuspend bacteria in 1 mL of water in a 24-well plate to an approximate OD_{600} = 5, perform two-fold serial dilutions, monitor OD_{600} of all dilutions, and plate each to determine the viable CFU/mL in each sample at multiple optical densities.

1.3. Determine the maximum sonication tolerance for each bacterial strain. To do this, aliquot cells into a 24-well plate containing liquid medium, reserving some cells as an unsonicated control sample. Using an ultrasonicator with a 24-tip horn attachment, apply three rounds of 12 s of sonication at 40 amp with 2 s pulses.

NOTE: The use of a 24-well ultrasonicator is advised to facilitate the downstream multiplexing of processing plant samples, but if one is not available, use an ultrasonicator fitted with a microtip and perform each sample sonication independently. Always wear earmuffs rated to at least 25 NRR protection.

1.4. Perform 10-fold serial dilutions of the sonicated and unsonicated samples and spot onto agar plates. Determine whether there is a reduction in viable cells after sonication. If so, use a fresh sample and repeat sonication step using a reduced total sonication time or amplitude until the treatment has no effect on final CFU/mL[17].

2. Preparation of *Arabidopsis thaliana* seedlings on a plastic mesh

2.1 Create disks of the plastic mesh using a standard hole puncher.

2.1.1. Collect the disks in a glass container with a loose cover of aluminum foil, and sterilize using an autoclave set to a 20 min dry cycle[13].

2.2.2. Using flame-sterilized tweezers, distribute approximately 40 sterilized mesh disks in a single layer across the surface of a plant-growth-medium agar plate. Use 0.5x Murashige and Skoog (MS) salts, containing 500 mg/L of MES buffer [2-(N-morpholino)ethanesulfonic acid] and 1.5% Bacto agar, as plant growth medium, with 50 µg/mL benomyl added to the limit fungal contamination of the seedlings.

2.2 Prepare axenic seeds of *A. thaliana* as previously described[17].

2.2.1. Place approximately 100–300 seeds each into individual centrifuge tubes in a rack and place into a resealable glass or heavy plastic container (“jar”) in a fume hood.

2.2.2. Using caution, place a beaker of 100 mL of bleach into the jar, add 3 mL of concentrated HCl to the bleach, and immediately seal the jar and allow fumes to sterilize seeds for at least 4 h.

2.2.3. Carefully remove the tubes of sterilized seeds from underneath the jar and seal.

2.3 Place two seeds at the center of each mesh. Seal plates with surgical tape and incubate for 2–6 days at 4 °C in darkness to vernalize seeds.

2.4. To germinate and grow seedlings, place the plate agar side down in a plant growth chamber for 8–10 days under short day settings: 9 h of light at 21 °C and 15 h of dark at 18 °C (**Figure 1,** step 2).

3. Colonization of plants in liquid bacterial growth medium

3.1 Add 1 mL of bacterial growth medium to each well of a sterile 24-well plate, except for media-only control wells. Use Lennox Luria Broth (10 g of tryptone, 5 g of yeast extract, 5 g of NaCl) as the bacterial growth medium.

3.2 Transfer the germinated seedlings embedded in mesh from agar plates to the liquid (**Figure 1,** step 3a).

3.2.1. Gently peel the mesh containing two germinated seedlings up and off the agar plate using flame-sterilized forceps. Choose mesh with equally sized and undamaged seedlings.

3.2.2. If removal from the agar is not smooth, discard that mesh and plant. Transfer one float to each well of bacterial growth liquid, root side down.

3.3 Inoculate bacteria into wells containing floating seedlings.

3.3.1. Resuspend bacteria grown overnight on agar plates to an OD_{600} equivalent to 10^8 CFU/mL in the bacterial growth medium liquid. Add 10 µL of bacterial suspension to each well for a final concentration of 10^6 CFU bacteria per well.

3.3.2.If preparing a mix of bacteria, resuspend each to the OD_{600} equivalent to 10^8 CFU/mL, mix in equal proportions, and add 10 µL of the final mix per well of liquid.

3.3 Seal the plate for sterile growth. Without touching the sticky side, carefully press the gas-permeable film across the plate. Ensure that each well has been individually sealed by applying pressure around each of the rings made by the wells. Replace the plate's plastic lid snuggly over the plate and gas-permeable film (**Figure 1,** step 3b).

3.3 Incubate the plates for 18 h in a plant growth chamber, under the same conditions as the seedlings were originally germinated, except on an orbital plate shaker set to 220 rpm.

4. Maintenance of bacterial colonization

4.1 To rinse all floats (plants on mesh), add 1 mL of sterile water to wells of a new 24-well plate. Remove gas-permeable film. Using sterile forceps, transfer floats to wells with water (**Figure 1,** step 4a). Rinse by resting for 10 min at room temperature (RT) without agitation.

NOTE: To determine bacterial colonization efficiency of roots rather than their ability to maintain colonization over time, plants can be sacrificed at this step by taking them directly to step 5.1.

4.2 Fill the wells of a new 24-well plate with 1 mL of plant growth medium. Transfer one mesh to each well. Cover with a gas-permeable seal and incubate for 72 h on the orbital plate shaker at 220 rpm in plant growth chamber (**Figure 1,** step 4b).

4.3 Repeat the rinsing as performed in step 4.1 with floats after the 72 h incubation period.

5. Collection of bacteria for viable cell counts

NOTE: The number of bacteria per seedling root can be determined at any incubation timepoint. Colonization can be monitored between 0 h and 18 h, while maintenance can be monitored from 18 h onwards. Plants destined for imaging can proceed directly to section 6.

5.1 Remove the seedlings from the mesh (**Figure 1,** step 5). Gently place flame-sterilized forceps below the leaves (but on the leaf side of the mesh), and lightly pinch the stem. Wiggle the seedlings up and away from the mesh to dislodge the root without breaking it. If the root breaks, gently scrape the mesh bottom to collect the full length.

5.2 Remove bacteria from plant roots. Transfer the bacteria to wells of a 24-well plate containing 1 mL of ddH_20. Sonicate the samples as described in step 1.3.

NOTE: Using a microscope, look for any remaining bacteria on the root surface on a sonicated sample. If bacteria remain, increase the total sonication time or intensity until no bacteria remain bound, up to the highest level of sonication that does not affect viable cell counts as determined in section 1.

5.3 Quantify the bacteria on roots.

5.3.1 Perform serial 10-fold dilutions of the sonicated samples up to a 10^{-6} dilution in bacterial growth medium. Add 50 µL of each dilution to individual agar plates and spread with sterile glass beads (or bacterial spreader). Incubate plates at the optimal temperature for bacteria until individual colonies are countable.

5.3.2 Once distinguishable, count the number of each colony morphology (as determined in section 1), and calculate CFU of each bacterial species per seedling. Discard any samples showing contamination, as contamination during colonization or maintenance may affect bacterial presence.

6. Collection of intact plant roots for microscopy

6.1 Using forceps, remove the seedlings from mesh as in section 5.

6.2 Transfer each plant to microscope slides.

6.2.1. Place the tip of the root on the slide and drag away from the tip to set the shoot down flush with the slide, ensuring a straightened root for best imaging. Add a drop of water or sterile plant growth medium to the samples to hydrate interfaces between the coverslips and slides.

6.2.2. Place a glass coverslip just above the root crown (uppermost boxed region in **Figure 1**) and below the shoot leaves to avoid slanting of the coverslip (to allow for root crown imaging), and press down gently[17].

6.3 If using fluorescent bacteria, image using appropriate excitation/emission filters to differentiate bacteria from each other and the plant root[18].

REPRESENTATIVE RESULTS:

The well-characterized PGPB *P. simiae* WCS417r is known to colonize the roots of *A. thaliana* in hydroponic culture. This naturally fluorescent bacterium can easily be visualized using microscopy on the roots of seedlings following colonization (**Figure 2**). Although it is possible to image the full length of these *A. thaliana* seedlings' (4–6 mm length) roots, doing so for many plants would take a prohibitive amount of time. Because most variation across timepoints and species of bacteria can be captured by imaging the crown, middle, and tip of the root[14] (indicated by red boxes in **Figure 1**), these regions were prioritized for imaging rather than imaging full root lengths. In the bright-field images of *P. simiae*-colonized *A. thaliana* roots (**Figure 2**)**,** it is possible to visualize the outline of the roots and root hairs; however, at 18 h of colonization, it is not possible to clearly differentiate colonized versus non-colonized roots using bright-field images. While *P. simiae* displays autofluorescence, we used a strain also engineered to express a yellow fluorescent protein (YFP)[19] with excitation/emission wavelengths of 490-510/520-550 nm[18]. A magnification of 100x was sufficient to clearly identify individual and small aggregates of *P. simiae* cells on *A. thaliana* roots. As shown in **Figure 2**, laboratories with access to either high-resolution confocal microscopes or less expensive benchtop microscopes can both visualize the presence and distribution of bacteria along the root.

While informative in terms of spatial distribution, microscopy images are not well-suited for quantification of bacterial cells. We thus collected bacteria from the surface of roots using ultrasonication as previously described and validated[9,20]. Three rounds of 12 s of ultrasonication[21] at an amplitude of 40 were sufficient to disrupt the outer surface of the root seedlings (**Supplemental Figure 1**) and remove all bacteria while not impacting the bacterial viability. Sonication was used rather than bead-beating methods[9] to better promote dispersal of bacterial aggregates/biofilms. Quantifying CFU/root after 18 h of colonization and an additional 72 h of maintenance showed that *P. simiae* both colonizes and is maintained on the roots of *A. thaliana* in our hydroponic, floating seedling system (**Figure 3**). The number of CFU/seedling at either timepoint showed good reproducibility across biological replicates

performed on different days (**Figure 3**). The variation observed is common among root colonization assays[22] and is likely due to minor variations of timing, environmental conditions, or plant root size, even among seedlings germinated at the same time and selected to be similar in size. We observe an increase in the number of CFU/seedling after 72 h in maintenance medium as compared to the numbers observed at the post-colonization 18 h timepoint (**Figure 3**). This indicates active growth of the colonized bacteria on the plant root occurred during the maintenance stage.

In addition to the utility of this hydroponic assay to quantify individual bacterial colonization and maintenance, it is also applicable to monitoring the association of multiple species on plant roots. To demonstrate this, three species of bacteria isolated from *A. thaliana* grown in natural soil under laboratory conditions were chosen[20]. The isolates were strains of *Arthrobacter nicotinovorans, Microbacterium oleivorans,* and *Curtobacterium oceanosedimentum*[23]. This simplified community was chosen due to these species' ability to coexist in liquid bacterial growth media in shaking culture (unpublished data). In addition, these three species can be clearly differentiated on media containing X-gal due to differences in colony morphology and color (**Figure 4A**). The X-gal does not affect relative growth of any of these bacterial species (unpublished data). These differences in morphology and colony appearance on X-gal allowed us to count the CFU/seedling of each species without antibiotic selection, even in multi-species coculture.

A. nicotinovorans, M. oleivorans, and *C. oceanosedimentum* were all colonized and maintained on the root, whether alone or in bacterial coculture (**Figure 4B**). Each species showed trends that were similar across different biological and technical replicates, even within mixed communities. This demonstrates that the assay protocol can be used to measure both relative or total CFU/root of each species. Interestingly, when grown alone, no individual species showed an appreciable increase in abundance during the maintenance stage, but the overall CFU/root of the combined community increased in cocultures, indicating that these bacteria do not prohibit the colonization of the other strains.

For all experiments, plants grown in liquid media without the addition of bacteria as negative controls were always included. No bacteria were visible on these control roots during microscopy (**Figure 2**), nor were any bacteria detected via plating for CFU (unpublished data). This indicates that sterilization of seeds and using the sterile techniques during this assay were sufficient to keep plants axenic unless purposely colonized.

FIGURE AND TABLE LEGENDS:

Figure 1: Assay for bacterial colonization and maintenance on *A. thaliana* roots. *A. thaliana* seedlings grown on sterilized plastic mesh were transferred to a growth medium optimized for bacteria [here, 0.1 x LB (Luria Broth) Lennox]. Bacteria then colonized the root over 18 h while the plant floated in shaking liquid. Following a rinse, the colonized float was transferred to a growth medium optimized for plants (0.5x MS + MES) for 72 h to test for maintenance of bacteria on the roots. The float was then rinsed, and the plant with any attached microbes was

removed for analysis (quantification of CFU/seedling or imaging by microscopy).

Figure 2: Visualizing *P. simiae* colonization of roots with fluorescent microscopy. *P. simiae* (false-colored green) colonized *A. thaliana* roots and was maintained on the root following transfer to plant-growth medium. Root crown (left), mid-length (center), and tip (right) at 40x magnification are shown from areas indicated in **Figure 1**. The top two rows show the bright-field and fluorescent images of roots colonized by *P. simiae* (imaged by epifluorescent microscopy). The same roots were also imaged by a confocal microscope (center two rows). The no-bacteria negative control in the two bottom rows showed no colonization. Scale bars represent 50 µm.

Figure 3: Quantification of *P. simiae* on *A. thaliana* roots. Total number of *P. simiae* viable cells recovered per *A. thaliana* seedling following 18 h of colonization or 72 h of maintenance. Three individual biological replicates are shown, each containing three technical replicates of two seedlings per float. The numbers shown are the means from the technical replicates, while bars represent standard errors of the means.

Figure 4: Quantification of the colonization and maintenance of a mixed bacterial community on *A. thaliana* roots. (A) Colonies of *A. nicotinovorans, M. oleivorans,* and *C. oceanosedimentum* can be differentiated on X-gal-containing agar medium by colony morphology and color. **(B)** Roots of 10 day-old seedlings were inoculated with approximately $3x10^5$ CFU/mL of each of the three strains. Shown are total CFU/seedling recovered of each species following 18 h of colonization or 72 h of maintenance when colonized either alone or in a three-member bacterial community. Two biological replicates, each comprising two technical replicates of two seedlings per float, are shown. The numbers shown are the means from the two technical replicates, while bars represent standard errors of the means .

Supplemental Figure 1: Ultrasonication disrupts the root surface. To dislodge bacteria from the surface of the root, whole plants were sonicated, and the bacteria was released into the liquid, which was serially diluted and plated for quantification of CFU/seedling. **(A)** An intact seedling is **(B)** structurally disrupted following ultrasonication.

DISCUSSION:

Plants in all environments interact with thousands to millions of different bacteria and fungi[5,7]. These interactions can either negatively and positively impact plant health, with potential effects on crop yield and food production. Recent work also suggests that variable colonization of crops by PGPBs may account for unpredictable plant size and crop yield in field trials[22]. Understanding the mechanisms behind these interactions might allow us to directly manipulate plant-associated microbial communities to aid in healthy plant development, even under stress[24].

Because bacterial colonization of roots and their maintenance in the rhizosphere is critical for plant-microbe interactions[9], we wanted to build a system to reproducibly visualize and quantify these bacterial behaviors. This hydroponic, floating seedling growth system allows for

392 microscopic imaging and quantification of bacterial populations on the roots of *A. thaliana*.
393
394 The described plant-microbe interaction assay integrates beneficial elements of existing
395 experimental protocols. The floating mesh method was based on that from Haney et al.[13],
396 which measured initial colonization of *P. simiae* WCS417r on static, floating *A. thaliana*
397 seedlings. In evaluating this system, strong colonization of *A. thaliana* roots by *P. simiae* was
398 validated, even though a different growth medium from Haney et al. was utilized and included
399 orbital shaking during colonization. The inclusion of orbital shaking during both colonization
400 and maintenance facilitates bacterial interactions that might not occur in static culture, as well
401 as reduce anoxic conditions that can inhibit both bacterial growth and plant root health[13]. We
402 also integrated aspects of microbiology-focused protocols designed to support plant root
403 colonization by various bacterial species[8,15,25]. This included a crucial transfer step out of the
404 medium optimized for bacterial colonization and into a medium optimized for plant growth.
405 This transfer to fresh medium also will allow the mechanisms underlying bacterial maintenance
406 on roots to begin to be addressed, an approach that may provide insights into the erratic
407 maintenance of PGPB in field trials[6].
408
409 This assay was optimized for rapid processing of multiple samples to allow for environmental
410 variables and mixed bacterial communities to be assayed within one multi-well biological
411 replicate. While ultrasonication has been previously shown to be sufficient for disruption and
412 collection of rhizosphere bacteria, the 24-well plate and multi-prong horn attachment quickens
413 sample processing. This cell viability calculation approach to quantifying bacterial presence
414 could be complemented by, or expanded to include, qPCR or MiSeq 16S rRNA gene community
415 profiling to determine the relative abundance of more diverse communities of colonizing
416 bacteria[20]. In addition, during imaging, the utility of focusing on just three regions of each plant
417 root to speed the visualization of bacterial presence and localization on the roots is highlighted.
418 The colonization of these different root regions has been shown to differ among bacterial
419 species[14]. Imaging can be performed with either naturally autofluorescent bacteria or
420 genetically tractable bacteria engineered to express a fluorescent protein.
421
422 The methodology described here allows for fast and reproducible evaluation of root
423 colonization by PGPB bacteria, but there are limitations to the conclusions that can be drawn
424 from these experiments. For instance, the ability for bacteria to chemotax towards the root is
425 known to be important for many bacterial species' colonization of plant roots, but this process
426 may not required within this shaking inoculation system. That said, for studies specifically
427 interested in chemotaxis, the colonization step could be performed in static liquid culture or on
428 the surface of a soft agar medium, where bacteria could be plated distant from the plant,
429 requiring them to actively move towards the root. In addition, a relatively rich growth medium
430 during colonization was used to promote bacterial growth and plant attachment; however,
431 these comparatively high nutrient concentrations may prohibit the examination of bacterial
432 utilization of or competition for plant-derived carbon during colonization. Again, depending on
433 the growth requirements of the bacteria being studied, the colonization medium can be varied
434 to best suit the particular research questions of specific researchers.
435

This system was designed to be easily amenable to different bacterial and plant growth conditions and to the addition of different environmental stressors and timepoints. However, the methods described here are best suited for measuring bacterial interactions with the roots of *A. thaliana* seedlings. Collection has been optimized for this plant, and larger or more sensitive plants may be intractable in the floating multi-well-plate system. Finally, while the bacteria of interest used here colonize the plant root in liquid culture, for other bacteria it may be necessary to inoculate the plant roots by dip-inoculation or plating on a solid agar medium instead[19].

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DISCLOSURES:

The authors have nothing to disclose.

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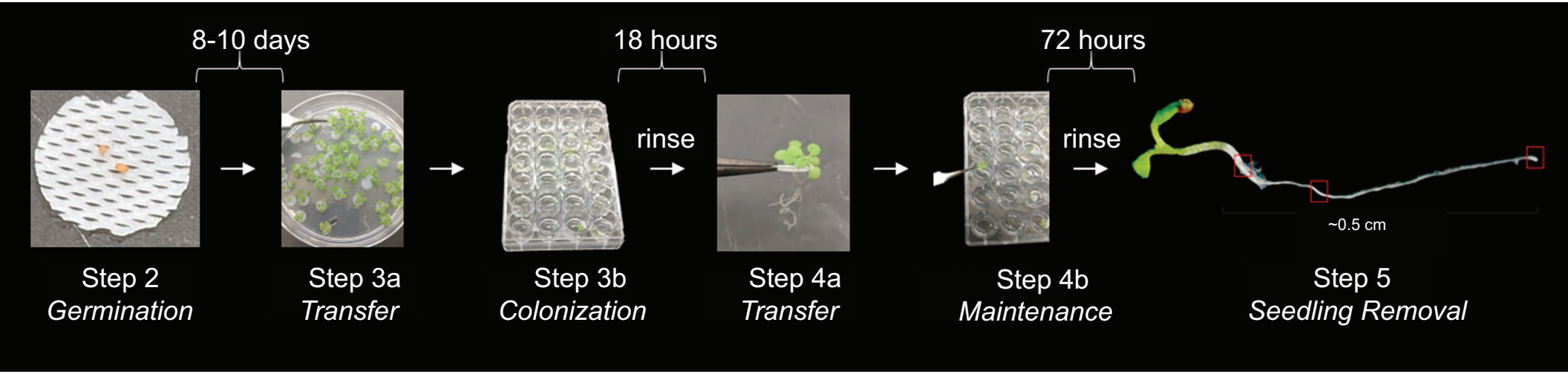
524

Figure



Figure

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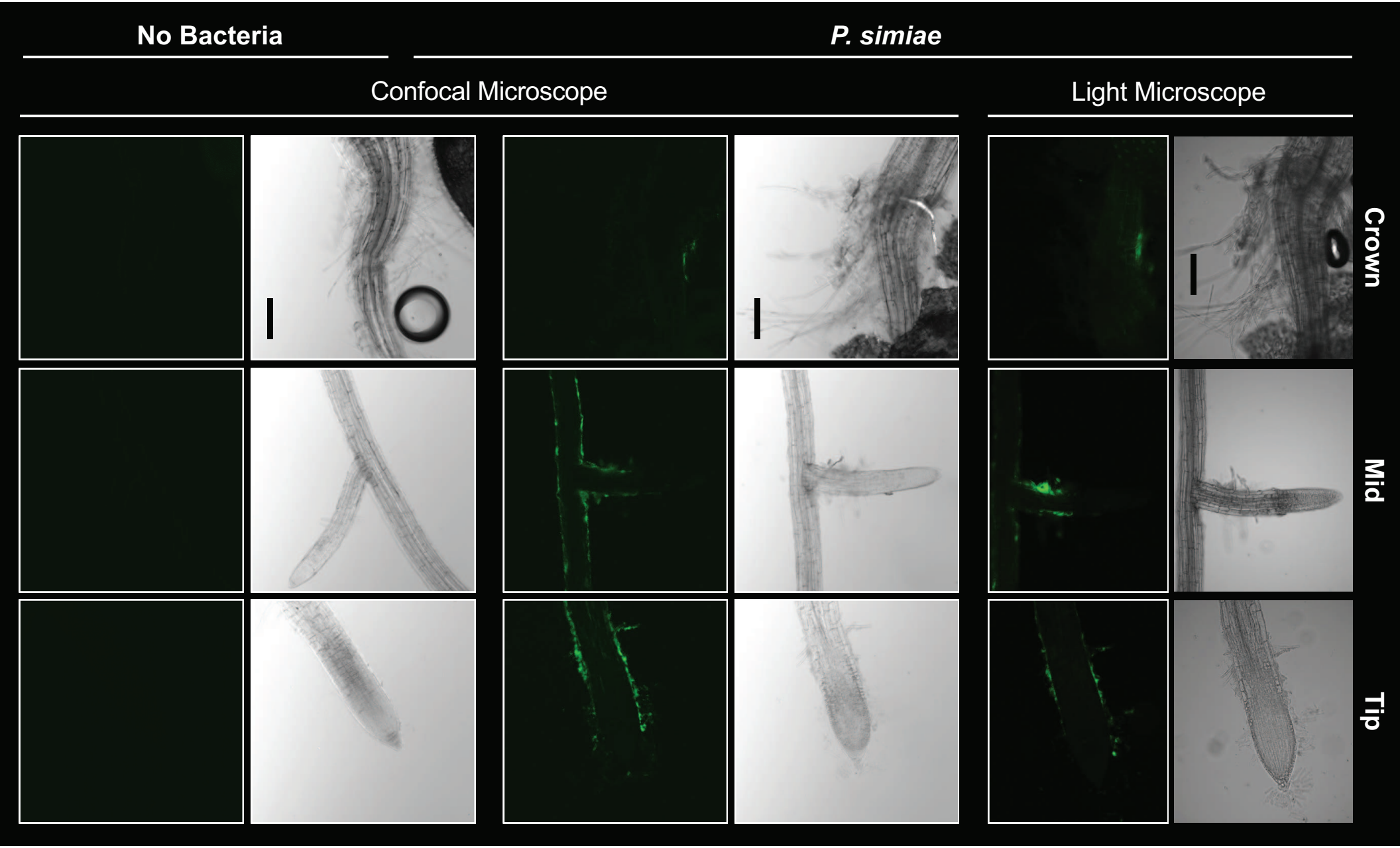

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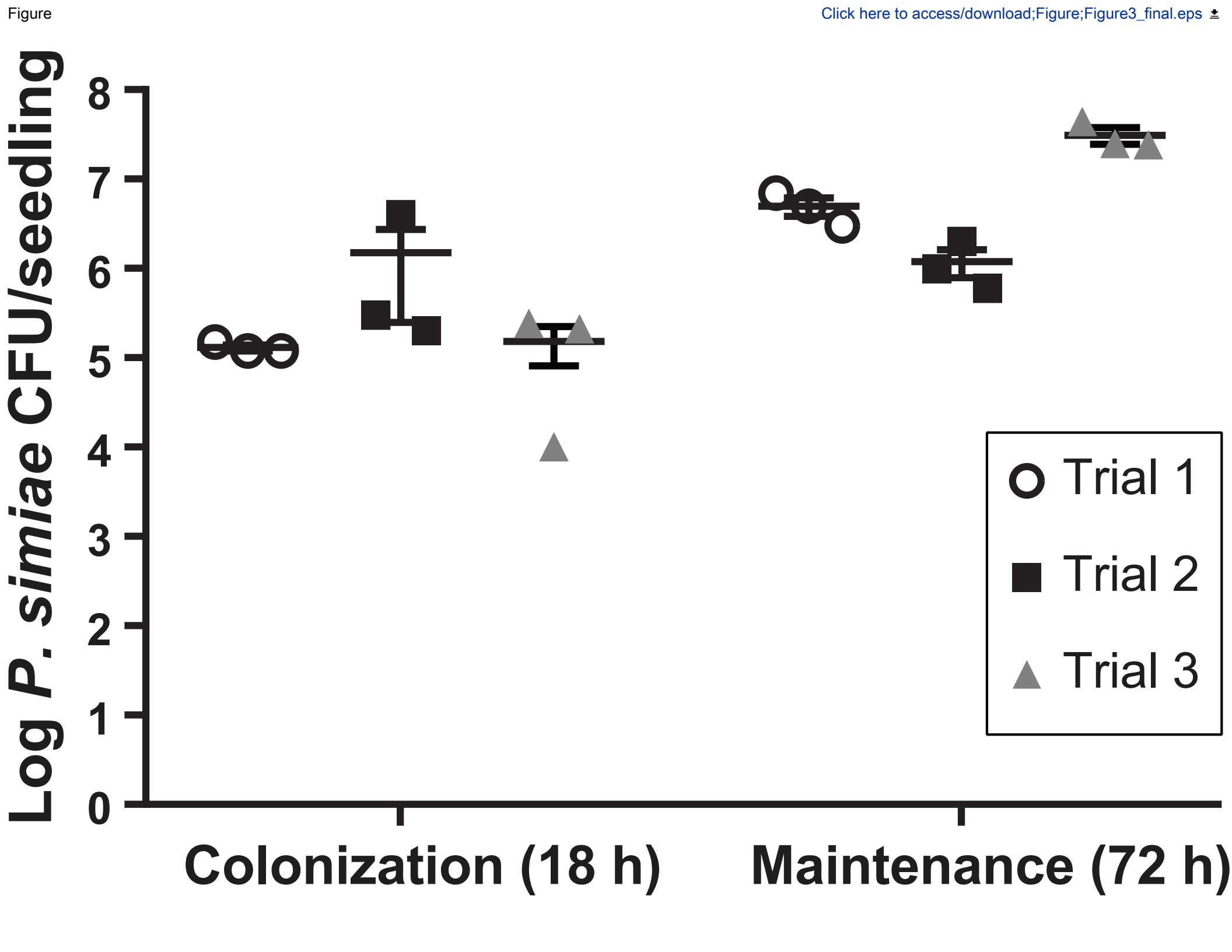

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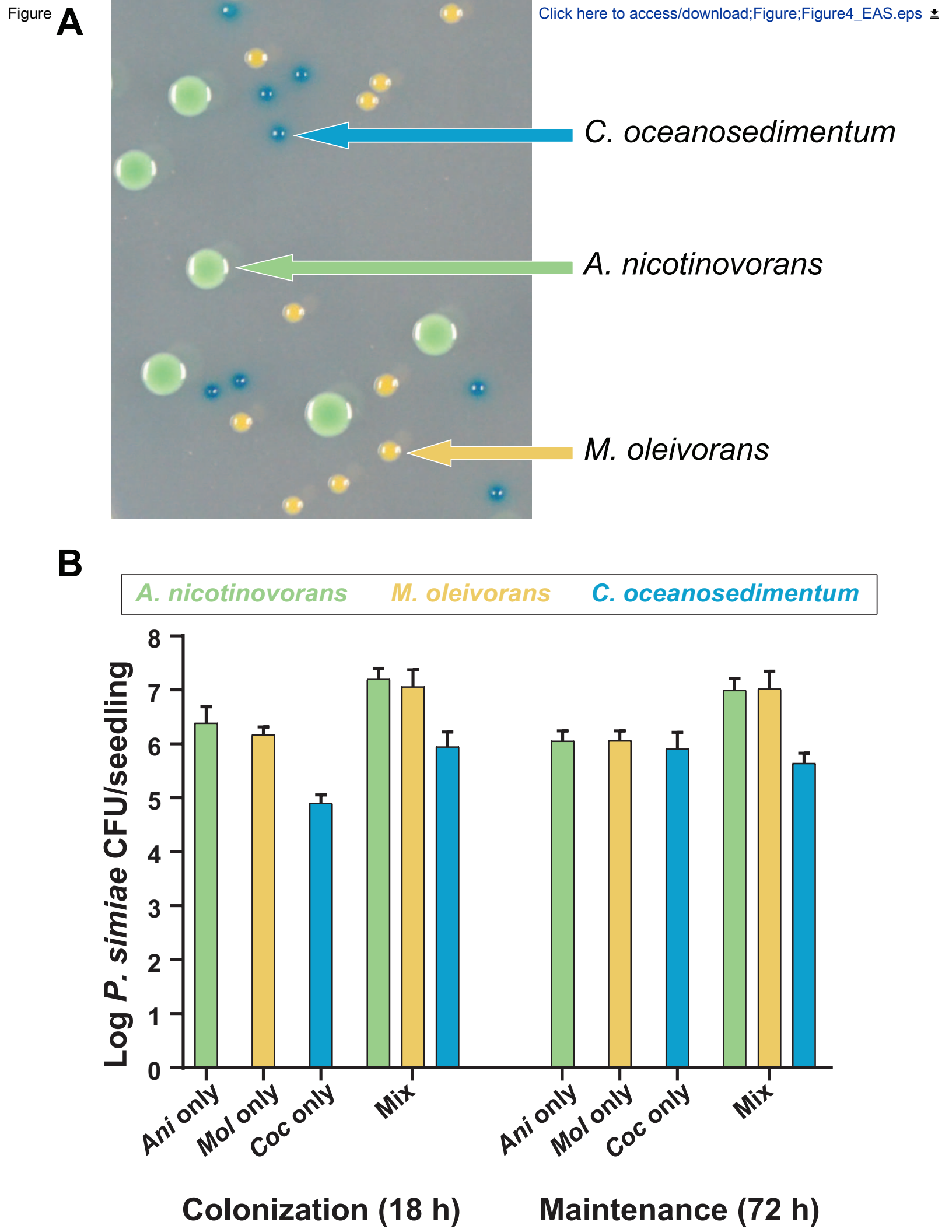

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Required Materials			
1.5 mL eppendorf tubes	any	N/A	
24-well plates	BD Falcon	1801343	
Aeraseal	Excel Scientific	BE255A2	
Autoclave	any	N/A	
Bacteria of Interest	any	N/A	Stored at -80˚C in 40% glycerol preferred
BactoAgar	BD	2306428; REF 214010	
bleach	any	N/A	
Conviron	any	N/A	Short Day Light-Dark Cycles: 460-600 µmoles/m²/s set at 9/15 hours light/dark at 18/21˚C, with inner power outlet
Dessicator Jar: glass or heavy plastic	any	N/A	
Ethanol	any	N/A	
Flame	any	N/A	
Forceps	any	N/A	
Incubator	any	N/A	At optimal temperature for growth of specified bacteria
Hydrochloric Acid	any	N/A	
Lennox LB Broth	RPI	L24066-1000.0	
Microcentrifuge	any	N/A	
Micropipetters	any	N/A	Volumes 5 µL to 1000 µL
Microscope (preferably fluorescence)	any	N/A	Could be light if best definition not important
MS Salts + MES	RPI	M70300-50.0	

Orbital Plate Shaker	any	N/A	Capable of running at 220 rpm for at least 96 hours
Petri Dishes	any	N/A	50 mL total volume
Reservoirs	any	N/A	
Spectrophotometer	any	N/A	
Standard Hole Punch	any	N/A	Approximately 7mm punch diameter
Sterile water	any	N/A	
Surgical Tape	3M	MMM1538-1	
Teflon Mesh	McMaster-Carr	1100t41	
Ultrasonicator	any	N/A	
Vortex Mixer	any	N/A	
X-gal	GoldBio	x4281c	other vendors available
	Suggested Materials		
24 Prong Ultrasonicator attachment	any	N/A	For sonicating multiple samples at once. Can be done individually
Alumaseal II	Excel Scientific	FE124F	
Glass beads	any	N/A	
Multipetter/Repetter	any	N/A	
Sterile 96-well plates	any	N/A	For serial dilutions. Can be replaced by eppendorf tubes
	Biological Materials Used		
Arabidopsis thaliana seeds	any	N/A	We recommend Arabidopsis Biological Resource Center for seed stocks
Arthrobacter nicotinovorans			Levy, *et al.* 2018
Curtobacterium oceanosedimentum			Levy, *et al.* 2018
Microbacterium oleivorans			Levy, *et al.* 2018

Pseudomonas simiae WCS417r	Published in a similar system in Haney, *et al.* 2015. Strain used developed in Cole, *et al. 2017*

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Author(s): Susanna L. Harris, Cesar A. Pelaez, Elizabeth A. Shank

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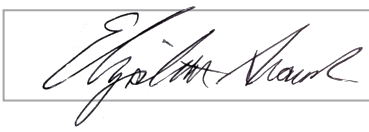
Signature:	 Date: 2018-12-07

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Dear Editor,

We greatly appreciate the opportunity to revise our manuscript ("Monitoring bacterial colonization and maintenance on the roots of *Arabidopsis thaliana* in a floating, hydroponic system") and thank both the editor and reviewers for their excellent suggestions to improve it. Below we describe the point-by-point changes that we have incorporated into our text and figures in response to these constructive comments. The editor's and reviewer's comments are in black text below, with our responses in red.

Editorial comments:

Changes to be made by the author(s) regarding the manuscript:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

We have now searched for and corrected grammatical and spelling issues.

2. Affiliations: Please provide an email address for each author.

Email addresses for each author have been added.

3. Please remove commercial language (Aeraseal, Nikon, etc.).

Commercial language has been removed in both text and figures.

4. Please revise the Protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

We have now edited the Protocol to remove all personal pronouns.

5. Please revise the Protocol to contain only action items that direct the reader to do something (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." Please include all safety procedures and use of hoods, etc.

Notes have been added to reflect necessary safety precautions and optional Protocol details. All Protocol steps are now actionable.

However, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Please move the discussion about the protocol to the Discussion.

Notes have been used to address limited, appropriate protocol details that are not actionable. The discussion-type text that is not necessary for completion of the Protocol has been moved to the Discussion.

6. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.

We have edited the Protocol to limit both actions and text length to the required format.

7. After you have made all the recommended changes to your Protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

We have highlighted the appropriate sections and length of the Protocol for videotaping.

8. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Notes cannot usually be filmed and should be excluded from the highlighting.

We have highlighted the appropriate section and length of the Protocol for videotaping.

9. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

We have highlighted the appropriate section and length of the Protocol for videotaping.

10. Figure 2: Please remove commercial language (Nikon, Zeiss).

As noted above, commercial language has been removed in both text and Figures

11. Figure 4: Please describe what panels A and B represent in the figure legend. Please move the details of the methodology to the Protocol section.

As noted above, commercial language has been removed in both text and Figures

12. Supplemental Figure 1: Please describe what panels A and B represent in the figure legend.

The figure legend has been amended to include this information

13. Please number the figures in the sequence in which you refer to them in the manuscript text. Currently, Figure 4 is referenced before Figure 2 and Figure 3 in the manuscript text.

Due to changes in the Protocol, all Figures are now referenced in the order in which they are numbered.

14. Table of Materials: Please sort the items in alphabetical order according to the name of material/equipment.

The Materials have been resorted to reflect alphabetical order, within the sub categories of: Essential, Recommended, Optional

15. References: Please do not abbreviate journal titles.

References have been changed to provide full journal titles for all citations.

Reviewers' comments:

Reviewer #1:

1). The title to reference 11 is incomplete. "A Primer on the ???"

Thank you for finding this error in the references. We have edited the text to include the full name of the citation paper: “A Primer on the *Arabidopsis thaliana* Model System”

2). For Figure 2, the root images shown are not DIC (differential interference contrast). The authors might want to refer to these as brightfield images instead of DIC

We agree that this is not the correct label and have edited both the figure and the text to reflect the correct description as brightfield.

3). For the naturally autofluorescent growth promoting bacteria mentioned in the article (page 5-

6), the authors might want to provide specifics of the excitation and emission wavelengths for optimal imaging of the bacteria.

Thank you for this note; we agree that this information would be helpful. We have adjusted the text to clearly convey the wavelengths at which *P. simiae* can be visualized using fluorescent microscopy on line 270.

Reviewer #2:

Major concerns

Line 389 - 403: The methodology described here allows indeed for fast and sturdy evaluation of root colonization by PGPR bacteria, but some the conclusions that can be drawn from this protocol have limitations which need to be discussed extensively. Shaking conditions during bacterial colonization does not allow experimenters to evaluate chemotaxis of the bacteria towards the root, which means that in this protocol, root colonization only results from the capacity of bacteria to attach to the root and form a biofilm. This is an important difference with natural conditions, in which chemotaxis towards plant is an important driver of colonization. Additionally, use of a bacterial colonization medium, and not of a carbon-deprived plant medium, will compromise the ability of experimenters to evaluate competition between bacteria for carbon sources around the root during colonization, and will also limit studies centered around interactions between bacterial species in the rhizosphere.

We agree that there are limitations to the described protocol. We have now added text (see lines 404-416 of Discussion) to clarify both that 1) there are limitations in comparing the results of this study to field-type environments, and 2) that many of the conditions and parameters of this protocol can be changed to better fit the needs of the researchers using it.

Minor Concerns:

The transition between line 107 and 111 is abrupt. Can each section of the protocol be justified/place in context very briefly?

We appreciate this being brought to our attention. The protocol has been changed to improve the logical flow between different steps.

111-112 : bacterial growth medium - why the uppercase letters?

Thank you for catching this remnant from an early draft of the manuscript. All words have been changed to lowercase form.

1.1.3 - This sub-section needs more details and needs to be put in a separate section (e.g. should be 1.2, not 1.1.3). It is an entire sub-protocol by itself.

We agree that the partitioning of the protocol was not as clear as it should be, so we have edited the text to better capture the discrete, actionable steps.

Line 148 : why the uppercase letters?

As noted above, all words have now been changed to lowercase form.

Line 150 : define MES - why is it necessary? is there a specific pH to this medium?

Thank you for pointing out the need for clarification here. The text has been amended to reflect that MES is a buffer.

3.2.1 Define more precisely "freshly grown". Is it overnight or 24h?

Thank you for this note, the wording has been changed to clarify this culture is grown overnight.

Line 232 : « bacterial spreader » instead of hockey stick

We agree and have changed the text to use this descriptor.

Line 265 : Imaging the full root can take a reasonable amount of time, depending on the type of microscope used - full length pictures and picture analysis could be used when possible (e.g. with an inverse fluorescence microscope).

This is true. We changed the text to indicate that many samples would be difficult to image in a timely fashion, but smaller sample sets would be feasible.

Line 293 : Variation could also arise from root integrity / health state of the plant.
Since variation might arise from difference in root length, the CFU/seedlings could be evaluated in CFU/mm of seedling root? That might reduce the difference between plants

We appreciate this suggestion, but because the *A. thaliana* seedlings are grown on agar plates and then transferred to liquid medium, these roots are generally curled and branching. Therefore, imaging or directly measuring each root would require manual manipulation that could affect CFU numbers.

Line 294 : selected

Thank you, we have now corrected this typo.

Title of Fig 3. on the graph : maintenance

Thank you, we have now corrected this typo.

Line 358, 360,361 : medium

Thank you, we have now corrected this typo.

Reviewer #3:

<u>Minor Concerns:</u>

In the introduction, claims that this system will help in addressing discrepancies between soil and laboratory conditions seem dubious, as the system proposed here is more tightly controlled and artificial than many laboratory studies.

We appreciate this perspective and have edited the introduction to better capture the goals of this *in vitro* growth system without overreaching.

Ln 123 - 'bacteria's' is incorrect

Thank you, we have now corrected this typo.

Ln 153 - if it can fit within length limits, it would be preferable to outline here these methods for preparing axenic Arabidopsis seeds.

We have now included a brief explanation of the vapor-phase seed surface sterilization method from the relevant citations.

Ln 166 - the numbered bullets skip 3.1.1

Thank you for notifying us, we have edited this error.

Ln 319 - is 'occlude' the correct word here?

We agree, and we have changed it to “prohibit”.

Ln 346 - 'A thaliana' should be 'A. thaliana'

We have corrected this error.

Ln 352, 359 vs. Ln 177 - why is a lower cell concentration used here vs. in the recommended protocol (10^5 vs. 10^6)

Thank you for notifying us. This was a typo on Ln 352/359, which has now been edited to correctly say 10^6

Ln 355, 368 - units differ slightly in the text vs. on the figures

Thank you for notifying us, the text has been edited to reflect the (accurate) data reflected in the figures.

Ln 363 - It is strange to reference the figure from within the figure legend.

We agree, and this has been changed.

Ln 365 - Are both technical replicates shown in the figure, then?

Yes, and the text has been edited to clarify this.

Figure 3 - The title over the chart should be removed. The name of the organism should be incorporated into the y-axis label. Lower error bar is missing in one treatment.

Thank you for the suggestions, we have changed the figure to accommodate these suggestions.

Figure 4 - The title over the chart in panel B should be removed. Lower error bars are missing for some treatments.

We have corrected the figure.

Supplemental Figure 1 - the legend does not explicitly describe the difference between panel A, panel B.

The Figure legend has been amended.

Supplemental Figure

Click here to access/download;Supplemental File (Figures, Permissions, etc.);Supplemental Figure Remade.eps