



Published in final edited form as:

Nat Protoc. 2021 May ; 16(5): 2450–2470. doi:10.1038/s41596-021-00504-6.

Peat-based gnotobiotic plant growth systems for Arabidopsis microbiome research

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Abstract

The complex structure and function of a plant microbiome is driven by many variables, including the environment, microbe-microbe interactions, and host factors. Likewise, resident microbiota may influence many host phenotypes. Gnotobiotic growth systems and controlled environments empower researchers to isolate these variables, and standardized methods equip a global research community to harmonize protocols, replicate experiments, and collaborate broadly. We developed two easily-constructed peat-based gnotobiotic growth platforms – the FlowPot system and the GnotoPot system. Sterile peat is amenable to colonization by microbiota and supports growth of the model plant *Arabidopsis thaliana* in the presence or absence of microorganisms. The FlowPot system uniquely allows one to flush substrate with water, nutrients, and/or suspensions of microbiota via an irrigation port, and a mesh retainer allows for the inversion of plants for dip or vacuum infiltration protocols. The irrigation port also facilitates passive drainage, preventing root anoxia. In contrast, the GnotoPot system utilizes a compressed peat pellet, widely used in the horticultural industry. GnotoPot construction has fewer steps and requires less user handling,

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Author Contributions

J.M.K, J.T and S.Y.H designed the FlowPot experiments. R.S and S.Y.H designed the GnotoPot experiments. J.M.K, R.S, B.P, D.R., C.T. and J.F. performed and/or analyzed the experiments. P.S.-L supervised J.M.K. during FlowPot protocol optimization at Max Planck Institute for Plant Breeding Research, Cologne. J.M.K, R.S, B.P, and S.Y.H wrote and finalized the manuscript with input from all co-authors.

Competing interests

The authors declare no competing interests.

thereby reducing the risk of contamination. Both protocols take up to four days to complete with four to five hours of hands-on time, including substrate and seed sterilization. In this protocol, we provide detailed assembly and inoculation procedures for the two systems. Both systems are modular, do not require a sterile growth chamber, and cost less than \$2 (USD) per vessel.

EDITORIAL SUMMARY

This protocol describes two peat-based plant growth systems for microbiome research in *Arabidopsis*. While both systems support microbe-free plants and input microbiota control, GnotoPots have advantages for throughput and FlowPots for versatility.

TWEET

A new protocol describing two peat-based plant gnotobiotic systems for microbiome research in *Arabidopsis*. #FlowPot #GnotoPot

COVER TEASER

Gnotobiotic systems for plant microbiome research

Keywords

Microbiome; gnotobiotic; phytobiome; germ-free; microbiota; axenic; holoxenic; *Arabidopsis*; dysbiosis; host-microbiota interactions

Introduction

Multicellular organisms are in constant contact with diverse microbial communities, which reside in and on their body parts. These microbes, collectively called microbiota, play crucial roles in their host health and disease¹⁻³. Plant microbiota is essential for maintaining plant health and promoting crop productivity, thus supporting human life and health. Much progress has been made to elucidate plant-microbiome interactions in open field or greenhouse conditions, but there are limitations to resolve the cause and effect of plant-microbiome interactions under such conditions^{4,5}. In particular, soils vary tremendously in their biochemical composition, microbial composition, and geochemical and physical attributes. Likewise, the air to which plants are exposed in open systems in different locations fluctuates in microbial composition and load. These variations are not amenable for a universally obtainable substrate for a global research community to replicate experiments easily. A deeper understanding of plant-microbiome interactions, especially the cause and effect relationship, is paramount to the use of microbiome for sustainable agriculture. This is a research priority for the plant microbiome community to develop standardized methods to elucidate the rules of microbiome assembly, functional plant-microbiome interactions, and isolate experimental variables to refine the understanding plant genotype-by-environment-by-microbiome-by-management interactions^{4,5}. Development of a set of gnotobiotic plant growth systems that can be widely adopted by the research community could facilitate the advancement of global plant microbiome studies. In previous studies, nutrient agar and calcine clay have been used in enclosed systems to investigate

plant-microbiome interactions⁶⁻¹⁰ (Table 1). However, these systems have limitations, as they do not contain organic matters and/or soil-like structure. Accordingly, development of plant gnotobiotic growth systems based on substrates that contain organic matter, such as peat-based potting mixtures, could more closely approximate a natural or agriculturally-relevant state.

Careful consideration must be taken when designing an axenic growth system to minimize artifacts and sample variability, but not at the expense of versatility. Environmental factors are the major drivers of differential taxonomic composition and diversity among host-associated microbiota¹¹⁻¹³. Thus, abiotic factors must be controlled to ensure reproducibility of observed host phenotypes and community dynamics. For example, in the model plant *Arabidopsis*, humidity greatly influences microbiota composition and the plant's ability to defend against foliar pathogens¹⁴. Other ecological drivers of soil microbiota composition are associated with physical and chemical attributes of the soil, including, but not limited to: pH, porosity and its gaseous composition, water retention, organic carbon, inorganic nitrogen and orthophosphate (Pi) availability, organic matter content, and cation exchange capacity^{15,16}. When conducting microbiota colonization experiments to compare gnotobiotic plants colonized with a known microbial community to axenic (germ-free) plants, it is essential to consider edaphic factors of the substrate as well as the growth environment, particularly if the experiment is intended to recapitulate the diverse microbial communities found in organic matter-rich soils^{4,5}.

Here, we present two axenic growth systems with peat-based substrate for plant microbiota research. These procedures allow for the growth of *Arabidopsis* in axenic (no viable microorganisms detected), gnotobiotic (inoculated with a defined community of bacteria), and holoxenic (inoculated with undefined microbiota extracted directly from a natural environment) conditions. Procedure 1 and Procedure 2 describe how to prepare and assemble the FlowPot and the GnotoPot systems, respectively. The FlowPot system has sterile peat substrate contained within a chamber. An irrigation port allows for precise control of watering and inoculation with suspensions of microbiota, nutrients and other inputs. Furthermore, FlowPots can be inverted while retaining substrate for dipping or vacuum-infiltration experiments. On the other hand, the GnotoPot system uses sterile peat wafers that can also be saturated with the same inputs as described for FlowPots. Their ease of GnotoPot construction may be conducive to experiments with higher throughput requirements.... We have recently used both systems in plant microbiota colonization studies for elucidating the significance of inter-kingdom microbial interaction in plant survival³ and establishing a causal relationship for phyllosphere microbial communities in promoting microbiota dysbiosis in plant leaves². While the FlowPot and GnotoPot systems were designed specifically for *Arabidopsis* growth, either system can likely be adapted to support the growth of other plants.

Development of the approaches

For the FlowPot system, we developed a protocol in which various substrates, including soil or peat mixes, could be used to support plant growth in a contained environment. Axenic plant growth requires that the substrate be sterilized, which in the case of peat,

can be accomplished by three autoclave cycles. Upon final assembly of the FlowPots, two successive aseptic flushing steps with water and with a nutrient solution will support plant growth^{2,3}. The Luer lock irrigation port at the base of the FlowPot supports subsequent irrigation, and the mesh substrate retainer allows researchers to invert the FlowPot for versatile microbial inoculation methods, including dipping and vacuum infiltration of leaves.

The GnotoPot system relies on using commercially available Jiffy-7[®] peat pellet substrate (manufactured by Jiffy Products, Norway, <https://www.jiffygroup.com/>). Since its introduction to market in 1967, the Jiffy-7[®] pellet which is prepared from *Sphagnum* peat moss and coir fibers, has been used extensively by hobbyists and professional growers for starting a wide variety of crops and ornamentals. We found that a hydrated Jiffy-7[®] pellet could be effectively sterilized by two autoclave cycles and that supplemental nutrients at the initial hydration step and final preparation step are sufficient to ensure healthy plant growth². Assembly of the GnotoPot system involves minimal handling steps, yet supports axenic plant growth in organic matter-rich plant growth substrate.

Overview of the procedures

Both procedures described here are based on predominantly peat-based substrates and utilize similar conditions for plant growth inside the same gas permeable Microbox tissue culture containers (SacO2, Belgium), however, the growth apparatus construction makes each procedure unique in terms of its potential applications and versatility.

For the FlowPot system (Procedure 1), each FlowPot (the gnotobiotic pot holding the substrate) is assembled using inexpensive and routinely available labware. In short, reusable components of the FlowPot system are constructed from truncated syringes (which form individual FlowPots bases) and modified pipette tip inserts (Steps 1-4). Soil or peat substrates are sterilized by autoclaving (Steps 5-7), then added to FlowPots bases, covered with a mesh retainer, and secured with a cable tie (Steps 8-10) (Fig. 1a). The FlowPot system features an inoculation port on each vessel (Fig. 1a) that enables substrate rinsing to remove soluble byproducts of soil sterilization, provides drainage, and accommodates homogenous inoculation with microbiota and/or nutrients. Assembled FlowPots are then autoclaved once more and aseptically irrigated from the inoculation port with nutrients and any desired input microbial suspensions (Steps 11-14)(Fig. 1b). Subsequently each FlowPot is placed into a sterile Microbox supported by a stand, and microbiota-free *Arabidopsis* seeds are sown on each FlowPot (Steps 15-16). The tissue culture boxes containing FlowPots are placed in a plant growth chamber with desired lighting and temperature conditions to support plant growth (Steps 17-18).

For the GnotoPot system (Procedure 2), the assembly of each unit begins with an initial hydration step of a compressed Jiffy-7[®] pellet. Here, a dry pellet is placed inside a small polypropylene pot and hydrated with a nutrient solution (Steps 1-2)(Fig. 2a). Next, GnotoPots are transferred to the Microboxes and secured in place with empty plastic pots (Steps 3-7)(Fig. 2b). Then, Microboxes containing GnotoPots are placed inside an autoclavable plastic bag, looselysealed, and autoclaved (Steps 8-12). At the final preparation steps (Fig. 2c,d), *Arabidopsis* seeds are sown aseptically on GnotoPots, desired input

microbiota communities are inoculated and the Microboxes are placed inside a tissue culture growth chamber with desired lighting and temperature conditions to support plant growth (Steps 22-26).

Features and applications of peat-based gnotobiotic systems

The gnotobiotic plant growth procedures presented here enable researchers to perform in-depth studies on the phenotype of axenic plants as well as plants colonized by soil-derived microbiota under highly controlled environmental parameters (Fig. 3 and Fig.4). These setups are effective platforms to study plant-microbiota interactions *in situ*, including microbial competitions within a synthetic root microbiota³ and determining the causal relationships contributing to leaf microbiota dysbiosis using microbiota transplantation experiments². Colonization of plants by synthetic or complex microbial communities can be performed at the seed stage or later during plant development via soil drench or other foliar applications.

The versatility of the FlowPot system facilitates the use of different soil and soil-like substrates for microbiota colonization studies. Additionally, using the mesh retainer allows FlowPots to be inverted for a variety of downstream applications, including dip inoculation or vacuum infiltration of aerial plant tissues with bacterial suspensions and other solutions.

Although the GnotoPot system is limited to the peat pellet substrate, the widespread availability of Jiffy-7[®] peat pellets make the GnotoPot system suitable for comparison of *Arabidopsis* microbiota colonization experiments across different labs. Furthermore, the larger size of the peat pellet substrate (relative to the FlowPots constructed in Procedure 1) supports plant growth for a longer period of time, making the GnotoPots system suitable for microbiota studies in fully expanded leaves during later plant developmental stages (Fig. 4). Lastly, the simplicity and minimal steps in the assembly of the GnotoPot procedure makes it feasible for an individual with minimal training to assemble more than 48 GnotoPots in 2 hours.

While the gnotobiotic systems described here have been designed for *Arabidopsis thaliana* root and phyllosphere microbiota research, we anticipate potential applications of peat-based gnotobiotic systems beyond *Arabidopsis* for growth of other model and non-model plant species under gnotobiotic conditions with minor modifications. However, this application will be limited to seedling and early vegetative growth stages of plants and will require the use of a larger gas permeable container (available from SacO2, Belgium) to accommodate larger plant species.

Comparison with other growth methodology for plant microbiome research

Axenic *Arabidopsis* growth can be accomplished with routine tissue culture methodology on a phytonutrient agar substrate (or similar) contained within a light- and gas-permeable container (see Table 1). However, routine tissue culture systems do not provide a soil-simulating growth substrate for microbial colonization. Furthermore, agar-based systems are notorious for non-uniform nutrient and O₂ delivery over time¹⁷. Hydroponic and aeroponic systems can alleviate issues with nutrient uniformity and O₂-delivery by agitation and media

replenishment, but such systems still do not provide a soil-simulating growth substrate for microbial colonization¹⁸. Furthermore, it can be challenging to maintain axenic conditions or prevent cross-contamination in common-reservoir hydroponic systems.

Non-soil substrates, such as sand, quartz, vermiculite, and calcined clay, are frequently used in gnotobiotic systems^{6,7,19-21}. These substrates are porous, thus providing varied surfaces for microbial colonization and root penetration. However, one major limitation of non-soil gnotobiotic systems is the lack of organic carbon typical of soil, which is beneficial to plant and microbial survival and/or growth. Furthermore, batch-to-batch variation of ceramic substrates can result in a wide range of labile ions²⁰. Calcined clay, for example, has sorptive properties that can reduce labile concentrations of P, Fe, Cu and Zn, and desorptive properties that can cause excess labile B, Mg, Ca, S, K, and Mn, leading to potential toxicity of the latter²². While thorough washing or soaking of the non-soil substrate can reduce the initial excess of labile ions, flow and drainage are important to reduce significant changes in chemistry over time. Nevertheless, compared to peat-based gnotobiotic systems described here, non-soil substrates, such as phytonutrient agar and calcined clay, may be suitable to mimic defined mineral nutrient deficiencies and other specific applications, thus highlighting the importance of multiple standardized gnotobiotic system methodologies.

Natural and agricultural soils have been used as substrates in axenic systems, but often present challenges, such as contamination or hindered plant growth due to suboptimal sterilization procedures. Numerous sterilization methods have been used with soil, including: autoclaving, dry heat, irradiation, microwave, fumigation by gaseous chemicals, and saturation with various sterilants²³. In our hands, soil sterilization methods need to be carefully optimized for plant growth due to potential phytotoxic effects of chemical residue and unintended artifacts of the sterilization process. Autoclaving soil has been shown to increase levels of water-soluble carbon, some ions and reduce pH²⁴, leading to potential nutritional imbalance, but not significantly alter ion exchange capacity. Gamma-irradiation has been reported to minimally disrupt the physical attributes of soils but can result in the generation of reactive oxygen species, capable of depolymerizing the C-C bond of polysaccharides²⁵. Both autoclaving and gamma irradiation can result in changes of the physical structure of the soil, exposing more surface area and thus altering sorptive properties. However, complete sterilization of some soils can be achieved with minimal chemical alterations by autoclaving a thin layer of soil for three short (<45 min) autoclave cycles with 18-24 hour intervals²⁶⁻²⁸. Subsequent flushing of sterile soil substrate can increase plant productivity, presumably by rinsing away soluble phytotoxic byproducts. However, in our hands, gnotobiotic systems based on heat-sterilized soils often do not provide a conducive environment for growing healthy Arabidopsis plants.

MATERIALS FOR PROCEDURE 1

Biological materials

- Arabidopsis seeds (from ABRC - Arabidopsis Biological Research Center; <https://abrc.osu.edu/>). See Box 2 for seed preparation, sterilization, and stratification and Box 3 for plant growth conditions.

- Sample containing input microbiota (e.g., from soil, see Box 1)

Reagents

- Sterile Milli-Q water (reverse osmosis filtered, or an equivalent quality water)
- Multi-Terge™ detergent (EMD Millipore, cat. no. 65068); diluted to 2% (v/v)
! CAUTION Multi-Terge detergent may be corrosive to metals and cause skin irritation. Use personal protective equipment as described by the manufacturer.
- Spor-Klenz™ disinfectant (Steris, USA, cat. no. 652026); diluted to 3% (v/v)
! CAUTION Spor-Klenz is a strong oxidizer and corrosive. Use personal protective equipment as described by the manufacturer.
- Linsmaier & Skoog (LS) medium buffered with 2-(N-morpholino) ethanesulfonic acid (MES) to pH 5.7 (Caisson Labs, cat. no. LSP03)
- Ethanol, 100% or 95% (v/v) (Fisher Scientific, cat. no. 04-355-451)
! CAUTION Avoid ignition sources and ensure proper ventilation when working with fire and flammable solvents such as ethanol....

Equipment

- Luer lock PP syringes, 50 mL (Jensen Global, cat. no. JG50CC-LL)
- Female Luer x female Luer adapter, nylon (autoclaved prior to use; Cole-Parmer, cat. no. EW-45502-22)
- Mesh fiberglass “Phiferglass”, 18 X 14 standard charcoal mesh (Phifer Incorporated, cat. no. 3003906)
- Soda-glass beads, 3 mm (Sigma-Aldrich, cat. no. Z265926)
- Microbox container (SacO2, cat. no., TP1600+TPD1200; #40 green filter, autoclavable)
- Filament tape model 893, 18 mm (Scotch Company)
- Redi-Earth plug and seedling mix (Sun Gro Horticulture, Canada). Contains fine Canadian sphagnum peat moss, vermiculite, dolomitic limestone, and a wetting agent
 - **! CRITICAL:** this can be substituted with alternative substrates, but plant performance may vary.
- Medium vermiculite, horticultural grade
- Polypropylene trays (United Scientific Supplies, cat. no. 81701)
- Sterilization wrap (Medline, cat. no. GEM1124S)
- Cable ties, 22 mm (TENAX Corporation, Baltimore, USA, cat. no. 120094)
- Sun bags (Sigma-Aldrich, cat. no. B7026)

- Cell strainer, 70 μm (Celltreat Scientific, cat. no. 229483)
- Drill bit, 8.8 mm (e.g., Chicago-Latrobe, cat no. 47329)
- Blocks of polypropylene, 12 cm X 8 cm x 1 cm (United States Plastic Corp, cat. no. 42605); alternatively, use Rainin RT-L1000 or similar tip box inserts

General equipment

- Biosafety cabinet or laminar flow hood (e.g., Logic+ Class II A2 Biological Safety Cabinet, Labconco, cat. no. 302611100; or Console Horizontal Airflow Workstation, Nuair, cat. no. NU-301-530)
- Test tube clamp or clamp modified hemostat (e.g., Stoddard Clamp, United Scientific Supplies, cat. no. TTCL03)
- Pipet and 1 mL filter tips (e.g., classic PR-1000 pipette and 1 mL RT-LTS filter tips, Rainin, cat. nos. 17008653 and 30389214)
- Funnel, 150 mm (Fisher Scientific, cat. no. 10-500-3)
- Glass Erlenmeyer flasks, 2 L (Corning, cat. no. 4980-2L)
- Sterile glass media bottles with screw cap, 2 L (Corning, cat. no. 1395-2L)
- Sterile graduated cylinders, 500 mL (Thermo Scientific, cat. no. 36620500)
- Bunsen burner (e.g., Humboldt Manufacturing Company, cat. no. H5870)
- Test tube racks (Thermo Scientific, cat. no. 59700020)
- Miter saw (e.g., Ryobi, cat. no. DC970K-2)
- Drill (e.g., 18-Volt Compact Drill/Driver, Dewalt, cat. no. DC970K-2)
- Growth chamber with desired lighting (e.g., Percival cat. no. CU36L5)

Reagent Setup

Multi-Terge detergent

- Dilute Multi-Terge concentrate to 2% (v/v) in water. Diluted detergent can be stored at room temperature (22-25°C) for several weeks.

Spor-Klenz disinfectant

- Dilute Spor-Klenz concentrate to 3% (v/v) in water. Prepare fresh solution daily.

LS nutrient solution

- Prepare LS solutions by dissolving the LS powder in water at 4.73g/L for a 1X solution. Autoclave the solution for 45 min. After autoclaving, cool down the media bottles to room temperature (22-25°C) then tighten the lid. Prepared LS nutrient solution can be stored at room temperature for at least three months. A 1X concentrate of buffered LS from Caisson Labs contains: NH_4NO_3 (1650 mg/L), H_3BO_3 (6.2 mg/L), CaCl_2 (332.2 mg/L), $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (0.025 mg/L), $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (0.025 mg/L), EDTA disodium dihydrate (37.26 mg/L), MES

(200 mg/L), MgSO₄ (180.7 mg/L), MnSO₄ · H₂O (16.9 mg/L), Na₂MoO₄ · 2H₂O (0.25 mg/L), Myo-Inositol (100 mg/L), KHCO₃ (98 mg/L), KI (0.83 mg/L), KNO₃ (1900 mg/L), KH₂PO₄ (170 mg/L), Thiamine hydrochloride (0.4 mg/L), ZnSO₄ · 7H₂O (8.6 mg/L).

MATERIALS FOR PROCEDURE 2

Biological materials

- Arabidopsis seeds (from ABRC - Arabidopsis Biological Research Center; <https://abrc.osu.edu/>). See Box 2 for seed preparation, sterilization, and stratification and Box 3 for plant growth conditions.
- Sample containing input microbiota (e.g., from soil, see Box 1)

Reagents

- Linsmaier & Skoog (LS) medium buffered with 2-(N-morpholino) ethanesulfonic acid (MES) to pH 5.7 (Caisson Labs, cat. no. LSP03).
 - Sterile Milli-Q water (reverse osmosis filtered, or an equivalent quality water)
 - Spor-Klenz™ disinfectant (Steris, USA, cat. no. 652026); dilute to 3% (v/v)
- ! CAUTION** Spor-Klenz is a strong oxidizer and corrosive. Use personal protective equipment as described by the manufacturer.

Equipment

- Biosafety cabinet or laminar flow hood (e.g., Logic+ Class II A2 Biological Safety Cabinet, Labconco, cat. no. 302611100; or Console Horizontal Airflow Workstation, Nuair, cat. no. NU-301-530)
- Standard Sterile Petri plates, 100 x 15 mm (VWR, cat. no. 25384-302)
- Jiffy-7® peat pellet, 36mm (Amazon, cat. no. B01LWMB93K)
- Small (2 inch) polypropylene nursery pots (Amazon, cat. no. B00LH1NMV0)
- Microbox container (SacO2, cat. no., TP1600+TPD1200; #40 green filter, autoclavable)
- Sun bags (Sigma-Aldrich, cat. no. B7026)
- 25 mL disposable serological pipets (Genesee Scientific, cat. no. 12-106)
- Electronic pipette controller (Scilogex, cat. no. 740200029999)
- Cell strainer, 70 µm (Celltreat Scientific, cat. no. 229483)
- Sterilization wrap (Medline, cat. no. GEM1124S)
- Pipet with 1 mL and 20 µL filter tips (e.g., classic PR-1000 pipette with 1 mL and 20 µL RT-LTS filter tips, Rainin, cat. nos. 17008653, 30389214, and 30389296, respectively)

- Growth chamber with desired lighting (e.g., Percival cat. no. CU36L5)

Reagent Setup

LS nutrient solutions

- Prepare LS solutions by dissolving the LS powder in water at 4.73g/L or 2.37g/L for 1X or 1/2X solution, respectively. Autoclave the solution for 45 min. After autoclaving, cool down the media bottles to room temperature (22-25°C) then tighten the lid. Prepared LS nutrient solution can be stored at room temperature for at least three months. A 1X concentrate of buffered LS from Caisson Labs contains: NH_4NO_3 (1650 mg/L), H_3BO_3 (6.2 mg/L), CaCl_2 (332.2 mg/L), $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (0.025 mg/L), $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (0.025 mg/L), EDTA disodium dihydrate (37.26 mg/L), MES (200 mg/L), MgSO_4 (180.7 mg/L), $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ (16.9 mg/L), $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ (0.25 mg/L), Myo-Inositol (100 mg/L), KHCO_3 (98 mg/L), KI (0.83 mg/L), KNO_3 (1900 mg/L), KH_2PO_4 (170 mg/L), Thiamine hydrochloride (0.4 mg/L), $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (8.6 mg/L).

Spor-Klenz solution—Dilute Spor-Klenz concentrate to 3% (v/v) in water. Prepare fresh solution daily.

PROCEDURE 1. THE FLOWPOT SYSTEM

Construction of FlowPots • TIMING ~1.5 h

! CRITICAL: In this procedure, a FlowPot is described as an individual growth vessel that contains peat substrate, and the final assembled setup consists of four assembled FlowPots within a Microbox. FlowPots are reusable. Steps 1-4 of the procedure only need to be performed for initial construction.

1. For each individual FlowPot, remove the piston from a 50 mL polypropylene (PP) Luer taper syringe. Using a miter saw with a fine-tooth blade, cut the syringe at the “20 mL” mark, retaining only the portion with the Luer connector. Mount the blade on the miter saw backwards for a smoother cut, and sand if needed. Remove any residual shards with a vacuum and a moist cloth. Soak the syringe tops for 20 minutes in 2% (v/v) Multi-Terge ionic detergent, and subsequently rinse the syringe top in Milli-Q water to remove all traces of the detergent. Autoclave prior to FlowPot construction.

! CRITICAL STEP Avoid syringes that have silicon oil or other lubricants within the barrel or wash thoroughly prior to initial use.

! CAUTION Use proper eye protection and keep hands out of the path of the blade when cutting plastic.

2. Cut 5 x 5 cm squares of mesh fiberglass. Autoclave prior to FlowPot construction.
3. Rinse 3 mm soda-glass beads 6 times with Milli-Q water. Dry and autoclave prior to FlowPot construction.

4. To construct a FlowPot stand, drill four holes in a 12 x 8 x 1 cm block of autoclave-compatible plastic (polypropylene or polycarbonate; e.g. disposable inserts from Rainin RT-L1000 or other pipette tip boxes) using an 8.8 mm drill bit. Orient the holes so they are evenly distributed with adequate spacing from stand edge so that the FlowPots do not exceed the stand boundaries.

! CAUTION Use proper eye protection and keep hands out of the path of blades and drill bits when cutting plastic and drilling inserts.

Sterilization of the substrate • TIMING ~3 d

- 5 Blend a 1:1 (vol:vol) ratio of peat potting mix and medium vermiculite (substrate). Moisten with Milli-Q water to achieve moisture content of approximately 60% moisture content. Evenly distribute the substrate on clean polypropylene laboratory trays at a depth of approximately 2 cm. Cover the surface of each tray with sterilization wrap in such a way that liquid will not collect on top during autoclaving and flow onto the substrate. Autoclave for 30 minutes on liquid cycle (121°C, 18 PSI, slow exhaust with forced liquid cooling) and bring to room temperature (22-25°C) immediately after autoclaving.

! CRITICAL STEP Do not let materials sit in the autoclave after cycling because this may cause the substrate to dry out, resulting in increased hydrophobicity and suboptimal plant growth.

- 6 Homogenize substrate in a sterile container and subsequently distribute on polypropylene laboratory trays. Let sit covered with sterilization wrap at room temperature for 24-48 hours.

! CRITICAL STEP The rest between autoclave cycles provides an opportunity for dormant microbial spores to germinate, which can then be killed during the second autoclave cycle.

- 7 Autoclave the substrate a second time (to kill any spores) for 30 minutes on liquid cycle (121°C, 18 PSI, slow exhaust with forced liquid cooling). Pre-clean the surface of a laminar flow hood using Spor-Klenz. Immediately after autoclaving, place the autoclaved trays of substrate in the pre-cleaned laminar flow hood and bring to room temperature. Once at room temperature, aseptically homogenize the substrate in the sterile laminar flow hood. Cover the trays of substrate with sterilization wrap. Leave covered at room temperature for 24-48 hours.

! CRITICAL STEP Depending on the moisture content of your substrate, relative humidity, and the calibration of your autoclave, autoclave parameters may need to be optimized to ensure sterility whilst preserving the integrity of the substrate.

! CAUTION Spor-Klenz is caustic and an eye/skin irritant. Use personal protective equipment as described by the manufacturer.

Assembly of FlowPots • TIMING ~2 h

- 8 Aseptically place 10 sterile glass beads (from Step 3) into each of autoclaved syringe tops (from Step 1). To stabilize FlowPots during assembly, use a sterile test tube rack (from Step 4). Gently fill each syringe top with the twice-autoclaved substrate mixture (from Step 7) until slightly heaping (~0.5 cm). Cover barrel end of the syringe top with the square mesh (from Step 2) and secure with a cable tie. Trim the excess edges of the square mesh.

! CRITICAL STEP Do not overpack the substrate. Compaction can lead to suboptimal plant growth. Within an experiment, it is critical to maintain the same relative compaction for all FlowPots.

! CRITICAL STEP: We recommend using a cable tie gun: Thomas & Betts Ty-Rap Tool (<http://www.cableorganizer.com/thomas-betts-ty-rap-tool.html>).

- 9 Once the test tube rack is full, place the test tube rack full of assembled FlowPots in a Sun bag and loosely close the end with autoclave tape such that the risk of contamination is minimized when the bag is removed from the autoclave, yet steam may still permeate the bag during sterilization. Autoclave for 30 minutes on liquid cycle (121°C, 18 PSI, slow exhaust with forced liquid cooling). Immediately after autoclaving, seal the opening of the Sun bag and move to a sterile hood.

! CRITICAL STEP Alternative autoclave-safe bags can be used instead of Sun bags. FlowPots can also be autoclaved directly in Microboxes as well, as long as care is taken to ensure steam can penetrate assembled FlowPots during autoclaving.

PAUSE POINT Sterile FlowPots inside sealed Sun bags can be stored for several days.

- 10 Center and fasten the drilled FlowPot stand to the inside bottom of a Microbox tissue culture vessel using filament tape. Autoclave constructed boxes and lids according to the manufacturer's instructions prior to use. Immediately after autoclaving, aseptically move to a sterile hood and place four assembled, autoclaved FlowPots into each Microbox.

! CRITICAL STEP We routinely use 18 mm filament tape model 893 (Scotch, USA), but alternative tapes are suitable.

PAUSE POINT Upon cooling, autoclaved Microboxes containing sterile, assembled FlowPots can be snapped closed and stored for several weeks.

? TROUBLESHOOTING

FlowPot irrigation and inoculation • TIMING ~2 h

- 11 Add 950 mL of sterile distilled H₂O and 50 g of sieved soil (see Box 1) to a sterile 2-L Erlenmeyer flask. Agitate soil slurry on a rotary shaker for 20 minutes at room temperature at 100-200 rpm, and subsequently let settle for 5

minutes. Filter the supernatant through a 40 μm cell strainer into a sterile 2-L Nalgene media bottle.

! CRITICAL STEP Allowing the slurry to settle increases reproducibility of colonization³¹ and reduces filter clogging.

- 12** Divide the soil slurry into two. Prepare the holoxenic inoculum by directly mixing the strained soil slurry with equal parts 1x LS media. To prepare a sterile mock inoculum, autoclave the remaining portion of the strained soil slurry for 45 minutes (121°C, 18 PSI, slow exhaust with forced liquid cooling), then mix with equal parts 1x LS media in a sterile laminar flow hood, bringing the final concentration of LS to 1/2x.

! CRITICAL STEP: The amount of inoculum needed for each condition will be determined by the number of FlowPots being prepared.

- 13** In a sterile hood, attach a sterile female Luer x female Luer adapter to a sterile 50 mL syringe. Using a flame-sterilized test tube clamp, grasp each FlowPot (from Step 10) and invert over a sterile funnel placed atop a waste flask. While inverted, use the sterile 50 mL syringe with attached adapter to aseptically infiltrate each FlowPot with 50 mL of sterile H₂O. Apply even pressure during the infiltration. After water infiltration, place the FlowPot back into its Microbox or on a sterile test tube rack. To reduce the risk of contamination, we recommend ethanol-flaming the test tube clamps between each FlowPot infiltration. The preparation of axenic FlowPots should be performed separately from those that are holoxenic and in a Biosafety cabinet or laminar flow hood.

! CAUTION Avoid ignition sources and ensure proper ventilation when working with fire and flammable solvents such as ethanol.

! CRITICAL STEP Occasionally, the glass beads become oriented in a way that the infiltration port is obscured. In this case, a sterile syringe needle may be inserted into the infiltration port to clear the blockage.

! CRITICAL STEP An alternative to the test tube clamp holder is a modified hemostat with semicircular stainless steel bands to grip the FlowPots.

? TROUBLESHOOTING

- 14** Let water-infiltrated FlowPots sit for 30 minutes, then infiltrate the FlowPots with 50 mL of a desired input community mixture (from Step 12). Evenly mix the input community prior to infiltration. Because of the small size of seeds to be placed on the surface of a prepared pot (see step 15), colonization of germinating plants is not expected to need inoculation of the entire system. For some other applications where a total saturation of the systems becomes necessary (e.g., study of microbial activities in different locations within a pot), an investigation of the evenness of inoculation within a pot will need to be conducted.

- 15** Place irrigation port of inoculated FlowPots in the drilled holes of the FlowPots stand within the sterile Microboxes. We recommend 4 FlowPots per Microbox for plants to receive even light coverage.

Sowing seeds • TIMING ~0.5 h

- 16** Aseptically sow approximately 8 seeds (see Box 2) per FlowPot using a pipette with filter tips.

! CRITICAL STEP Prepare surface-sterilized seeds using information from Box 2.

Plant growth • TIMING Up to 4.5 weeks

- 17** Place Microboxes with planted FlowPots in the plant tissue culture growth chamber (see Box 3).

! CRITICAL STEP After sowing, make sure the Microbox lids are completely sealed to maintain consistent humidity and sterility.

- 18** Aseptically thin boxes to 3 plants per pot using flamed forceps 7-10 days after germination.

! CRITICAL STEP Check sterility of plants and substrate during plant growth by using information from Box 4.

! CRITICAL STEP The syringe barrels can be reused after the experiment is completed to construct new FlowPots. In order to do so, discard contents, rinse thoroughly, and autoclave before storage or new FlowPot construction. ...

? TROUBLESHOOTING

PROCEDURE 2. THE GNOTOPOT SYSTEM

Preparation of the GnotoPot system (day 1) • TIMING ~45 min

! CRITICAL In this procedure, a hydrated Jiffy-7[®] peat pellet inside a plastic pot is referred to as a GnotoPot, and the final assembled setup consists of four GnotoPots within a Microbox. This procedure is for preparing 48 GnotoPots for within 12 Microboxes.

- 1.** Place 48 plastic nursery pots inside a large autoclave bin and add one dry compressed Jiffy-7[®] disc per pot.
- 2.** Add 3 L of freshly prepared ½ X LS nutrient solution to completely hydrate the pellets. Wait for 30-40 min. The pellets will expand to seven times the original height of the dry discs.

? TROUBLESHOOTING

- 3.** While pots are being thoroughly soaked in nutrient solution add a small piece of labelling tape to 12 Microbox containers on the side with an opening crack for labelling each box at the time of the experiment.
- 4.** Transfer two empty plastic pots to the center of each Microbox container.

5. Transfer four fully hydrated GnotoPots to each Microbox flanking the central empty pots (Fig. 2b).
6. Add 50 mL of the excess nutrient solution from hydration autoclave bin to each box.
7. Loosely place the Microbox container lid on top of each box. Do not snap close the lids.
8. Transfer two assembled Microboxes with loose lids into an autoclavable bag (Sun bag) and close the bag with a piece of labelling tape after folding the opening of the bag inside and then down.
9. Place assembled bags into an autoclave bin, cover with sterilization wrap and secure the wrap with binder clips.

! CRITICAL STEP Make sure that the final assembled setup does not go through any strong mechanical disturbances since the pots will tip over and would not be usable after the autoclave cycles.

Sterilization of the GnotoPot system (day 1 and day 3) • TIMING ~3 days

- 10 Autoclave the assembled setup from Step 9 for 45 min using a liquid cycle at 121°C (18 PSI). After the autoclave cycle is done allow the chamber to cool down to 50°C. Then store the assembled setup at room temperature (22-25°C).

- 11 After two nights (36 h to 48 h) repeat the autoclave cycle as Step 10 and cool down at room temperature.

! CRITICAL STEP The rest between autoclave cycles provides an opportunity for dormant microbial spores to germinate, which can then be killed during the second autoclave cycle.

- 12 After 3-4 h or sufficient cool down remove the sterilization wrap and snap close the lids while inside the Sun Bag. Store the Microboxes containing GnotoPots while inside Sun Bags at room temperature for at least 1 day before sowing seeds (Step 23).

PAUSE POINT At this stage, Microboxes containing GnotoPots inside the Sun bags could be stored away for future use up to at least two months without any contamination issues.

! CRITICAL STEP If the external surfaces of the Microbox lids have significant condensation after 1 day, allow more time for drying before use.

Preparation of the complex holoxenic community (day 4) • TIMING ~3 hours

- 13 Use the soil material prepared based on information in Box 1. To extract soil microbial communities, transfer 10 g of the soil aliquot to an autoclaved 1L Erlenmeyer flask and add 200 mL of autoclaved Milli-Q water.
- 14 Place the flask in a shaker for 20 min at 100-200 rpm at room temperature.

- 15 Store the flasks on the bench for 5 min allowing for separation of large soil particles from soil slurry.
- 16 Filter the soil slurry through a cell strainer and split into two 100 mL aliquots inside 1 L round media storage bottles.
- 17 Add 100 mL of autoclaved 1X LS to 100 mL of soil slurry for viable microbial community inoculation.
- 18 Autoclave the second half of soil slurry for 45 min for heat-killed community control. After cooling down to room temperature, place the heat-killed community inside a biosafety cabinet and add 100 mL of autoclaved 1X LS solution.

Transferring Microboxes containing GnotoPots to a biosafety cabinet (day 4) • TIMING ~30 min

- 19 To surface sterilize the interior environment of the biosafety cabinet use the germicidal UV lamp for 10 min. After lifting up the sash spray and wipe out all the working areas with freshly prepared Spor-Klenz solution.
 ! **CAUTION** Spor-Klenz is caustic and an eye/skin irritant. Use personal protective equipment as described by the manufacturer.
 ! **CRITICAL STEP** To ensure that the GnotoPots remain axenic during the handling steps, thorough aseptic practice is recommended.
- 20 Spray all the items to be used inside the biosafety cabinet with freshly prepared Spor-Klenz solution and store inside the biosafety cabinet.
- 21 Remove the Microboxes containing GnotoPots from Sun Bags and move the bags out of the biosafety cabinet.
 ! **CRITICAL STEP** in cases that the external surfaces of the Microbox lids are still wet at this point, allow the boxes to dry out further inside the biosafety cabinet before handling.

Seed sowing on GnotoPots (day 4) • TIMING ~45 min

- 22 Open each Microbox and add 15 mL of 1X LS solution to individual GnotoPots by top irrigation of the pots to restore the Jiffy-7[®] pellet water content to full saturation. At this step label each Microbox with appropriate information for different plant genotypes and treatments.
- 23 Using a P20 pipette transfer 1 or 2 seeds (see Box 2) per pot to the edge of the central divot of the GnotoPots.
 ! **CRITICAL STEP** To achieve a uniform plant growth in this procedure only one seed per pot is being used, therefore it is important to make sure that the seeds are of high quality with high germination rates following the steps described earlier (see Box 2).

- 24 For axenic plants with no further treatments snap close boxes and move them out of the biosafety cabinet.

Inoculation with microbial communities (day 4) • TIMING ~15 min

- 25 To inoculate with live complex microbial communities or synthetic bacterial communities² gradually irrigate the top section of pots with 1 mL of the microbial community solution (from Step 17) in a dropwise manner covering the entire top surface. For axenic plants treated with heat-killed community as a control uniformly add 1 mL of autoclaved and cooled down solution from Step 22 to the top section of GnotoPots. Then snap close the lids and move boxes out of the biosafety cabinet.

Plant growth • TIMING Up to 6.5 weeks

- 26 Move the GnotoPots to a plant tissue culture growth chamber (see Box 3).
! CRITICAL STEP Check sterility of plants and substrate during plant growth by using information from Box 4.
 ? TROUBLESHOOTING

Sampling GnotoPots for contamination tests • Timing ~30 min for six Microboxes containing GnotoPots

- 27 Test for sterility before using germ-free plant material for experiments by sampling the peat pellet from each GnotoPot for microbial contamination. Spray germ-free boxes with Spor-Klenz and store under the biosafety cabinet for 10 min. Then open up the boxes and, using a sterile loop, scoop a small amount of peat pellet from any part of the GnotoPots including the central hollow core and process samples using instructions from Box 4. Alternatively, test standing liquid inside Microboxes for contamination.
! CAUTION Spor-Klenz is caustic and an eye/skin irritant. Use personal protective equipment as described by the manufacturer.
! CRITICAL STEP Make sure that sampling is done without damaging the plant material or contaminating the system. Use this sample in the next step.

Troubleshooting

The FlowPot system requires more steps to construct and implement than the GnotoPot system. It is important to minimize the variability associated with construction of FlowPots (Procedure 1 Steps 1-4), and several attempts may be required to optimize Procedure 1. Table 2 summarizes several troubleshooting suggestions to help facilitate the optimization process. Table 1 also contains several troubleshooting tips for the GnotoPot system as well.

Timing

Any required soil or seed preparation should be performed prior to the start of the experiment.

Procedure 1 for preparation of 12 FlowPots

Pre-experiment preparation: Steps 1-4, construction of 12 FlowPots (3 FlowPot boxes): ~1.5 h

Days 1-4: Steps 5-7, sterilization of the substrate: ~3 d

Steps 8-10, assembly of FlowPots: ~2 h

Steps 11-15, FlowPot irrigation and inoculation: ~2 h Steps 16, sowing seeds: ~0.5 h

Procedure 2 for preparation of 48 GnotoPots

Days 1-4: Steps 1-12, preparation of the 48 GnotoPots (within 12 Microboxes) and two autoclave cycles: 3 d

Steps 13-18, preparation of the complex holoxenic community: ~3 h

Step 19-25, soil slurry and heat killed community preparation, and seeds sowing in GnotoPot: ~4 hours

Anticipated Results

We have shown that the FlowPot and the GnotoPot systems can be used to study microbiota dynamics *in planta*^{2,3} under a controlled environment using plants grown in organic matter-based peat substrates that more closely approximates a natural state as compared to other existing methods (Fig. 3 and Fig. 4). We expect that both systems will enable researchers to investigate a variety of questions related to the plant-microbiota interactions, ranging from community assembly to epigenetic impacts on the host, which will undoubtedly provide new insights in plant and microbial sciences. Specific scientific objectives may necessitate modification of either the FlowPot or GnotoPot systems, however, the procedures described here provide a strong foundation for future research.

The FlowPot and GnotoPot systems can be adapted to suit many research applications, including microbiota colonization studies as well as studies to determine the phenotypic impact different microbiotas may have on the host plant. A fundamental attribute of both systems is the ability to support the robust growth of axenic *Arabidopsis* plants. To demonstrate this feature, we performed Procedure 1 and Procedure 2 to produce axenic and holoxenic *Arabidopsis* plants in the FlowPot system (Fig. 3) and the GnotoPot system (Fig. 4). Briefly, FlowPots and GnotoPots were constructed according to the procedures described in this Protocol, and subsequently treated with a soil slurry or mock-treated with the heat-killed soil slurry. The soil slurry was generated from soil we collected from an agricultural field located at Michigan State University (East Lansing, MI, USA) (Supplementary Table 1). *Arabidopsis* seeds were sown, and plants were grown for 4 weeks (Fig. 3) or 6.5 weeks (Fig. 4) under the recommended growth chamber conditions. To prevent overcrowding, each FlowPot and GnotoPot was aseptically thinned to a single plant using sterile forceps. The axenic plants were verified to be axenic using the procedure described in this Protocol, and representative images of mature rosettes were photographed (Fig. 3 and Fig. 4). Plant samples from both systems could be subsequently used for various

plant phenotyping experiments and microbial community studies to determine the functional impact of microbiota on the host, microbiome composition, and to identify microbial strains that most successfully colonize host plant tissues.

One of the fundamental questions regarding host-microbiota interactions across different domains of life is how a host shapes its microbiome, and ultimately, how does this promote health and disease. The FlowPot and GnotoPot systems provide researchers with tools to address such questions. For example, Xin et al. (2017) noticed that two *Arabidopsis* polymutants deficient in aspects of pattern-triggered immunity and defense-associated vesicle trafficking (*min7/bak1/bkk1/cerk1* and *min7/fls2/efr/cerk1*) (referred to *mbbc* and *mfec*, respectively) developed spontaneous disease symptoms in the absence of pathogen inoculation under high humidity when grown in growth chambers with conventional potting mix substrate, which contains natural microbiota. To determine whether the phenotype was microbiome-dependent, the GnotoPot system was deployed by Chen et al. (2020), who performed an experiment to study leaf phenotypes in wildtype vs *mfec* *Arabidopsis* in the presence or absence of microbiota. Only holoxenic *mfec* had clearly visible chlorosis, demonstrating the microbiota-dependent nature of the phenotype. We repeated this experiment using the *mbbc* polymutant and observed a similar result (Fig. 5). Collectively, the GnotoPot system enabled experiments to demonstrate a causal role of microbiota in the observed spontaneous leaf damage associated with dysbiosis. We anticipate the use of the FlowPot and GnotoPot systems will enable researchers to broadly address questions about various microbiota functions.

The FlowPot and GnotoPot systems can also be used to determine the relative success of different microorganisms for their ability to colonize and persist in host tissues. Critically, MicroBoxes allow isolation of samples/treatments within a common growth chamber, preventing microbiota cross-contamination and thus allowing researchers to use multiple input microbial communities in a single experiment with limited space. Both defined microbiotas (for example, synthetic communities) and undefined natural microbiotas (for example, a soil slurry) can be used as input microbiotas in either system. The FlowPot system homogeneously saturates entire pots with input microbial suspensions (Fig. 1), while the GnotoPot system allows experimenters to apply microbial suspensions to the surface of Jiffy-7 pellets (Fig. 2). Here, we provide an example of a colonization workflow (Fig. 6). Using three distinct soils as input microbiotas (Supplementary Table 1), we inoculated FlowPots, collected whole *Arabidopsis* rosettes after four weeks of growth, and surveyed the bacterial community composition of rosette tissue (Supplementary Methods) to identify the most abundant bacteria present. The 16S *rRNA* gene operational taxonomic units (OTUs) that account for >2% of total reads in any given sample are displayed as a heatmap (Fig. 6), and closest-match OTU classifications at the genus level were also determined for this example application (Supplementary Table 2). Applications similar to this community profiling experiment have broad utility, not limited to 16S *rRNA* gene community profiling. For example, Duran et al. (2018) successfully used this approach to characterize fungal and oomycete communities associated with plant tissues.

With all animal and plant gnotobiotic systems there is always the challenge of keeping the enclosed system devoid of any unwanted microbial colonizers. The protocols described here

are designed to effectively eliminate substrate- and air-borne microorganisms without using antibiotic treatments and exclude microbes from the gnotobiotic systems prior to the start of gnotobiotic experiments in a cost-effective way. By using best practices of sterile techniques along with recommended troubleshooting steps included in this paper, we expect that the FlowPot and the GnotoPot systems could be successfully adapted by the plant-microbiota research community.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We would like to thank Caleigh Griffin, Alec Bonifer, Alan Mundakkal, Franchesca Dion, Trevor Ulrich, Jennifer Martz, and Tim Johnson for assistance with FlowPot assembly and workflow optimization, Dr. M. Amine Hassani and Dr. Stephane Hacquard for critical reading and helpful comments on this manuscript, and Dr. Brian Kvitko and Dr. JP Jerome for their contributions to FlowPot growth system development. Figure 2 was partially created with [Biorender.com](https://biorender.com). This project was supported by funding from Gordon and Betty Moore Foundation (GBMF3037), National Institutes of Health (GM109928), and Plant Resilience Institute, Michigan State University.

Data availability

All data are presented in this paper and available from the authors without restrictions. Information about the input soil microbial communities is available in Supplementary Table 1. Raw source 16S rRNA gene sequences from this project (for Fig. 6 and Supplementary Table 2) are available in the Sequence Read Archive database under BioProject PRJNA689857, accession numbers SAMN17220890 to SAMN17220933.

Related links

Key references using this protocol:

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Box 1 |**Preparation of the soil extract source microbiota**

This box provides information on how to collect and store soil for extraction of complex microbial communities. Soil collection is done when the soil has not recently experienced extreme conditions. Procurement of a source microbiota can be performed in advance of the experiment and modified depending on the input community characteristics and experimental parameters of your choosing.

Equipment:

- Whirl-Pak sterile sampling bags (Nasco, cat. no. B01065WA)
- 3 mm galvanized steel screen

Collection method

Collect and store soil using the following steps:

1. Remove topsoil (typically 10-15 cm) including any vegetation. At the sites of our soil collection, this helps to avoid variability of surface debris and organic matter. However, less topsoil may be removed if soil surfaces are less variable in debris and organic matter. Then collect more than 5 cm deep soil and transfer to the lab.
2. Let the soil sit for 1 week at room temperature (22-25 °C) with ~50% relative humidity.
3. Next, sift through a 3 mm galvanized steel screen to remove large debris.
4. Aliquot soil in 100 g increments and store at 4°C in Whirl-Pak bags.

Box 2 |**Seed preparation, sterilization, and stratification • Timing 3 d**

Arabidopsis seed sterilization can be performed via a variety of methods²⁹. Here we describe vapor phase sterilization as it allows high-throughput processing of multiple aliquots of different genotypes at once and can be performed ahead of time. To promote uniform germination and plant growth, seeds are first selected based on size using a sieve. Additionally, proper seed storage and cold stratification help ensure higher germination rates. As FlowPot and GnotoPot systems can be used to study plant interactions with vertically transmitted endophytes, specialized steps, such as antibiotic or fungicide treatment before seed harvest in the prior generation insect-free growth chambers, will need to be developed.

Reagents:

- Arabidopsis seeds (from ABRC - Arabidopsis Biological Research Center; <https://abrc.osu.edu/>)
- Bleach (common household bleach, 5.25% sodium hypochlorite (wt/vol); e.g. Clorox)

! CAUTION Bleach is corrosive. Use protective equipment.

! CRITICAL Use freshly opened bleach.

- Hydrochloric acid (HCl, 37% (vol/vol); Sigma-Adrich, cat. no. 320331)

! CAUTION HCl is corrosive. Use protective equipment.

Equipment:

- Metal Sieve, US Standard 60 mesh (250 μ m) (Fisher Scientific, cat. no. AA412000N)
- Metal Sieve, US Standard 50 mesh (300 μ m) (Fisher Scientific, cat. no. AA399850N)
- Microcentrifuge tubes (USA Scientific, cat. no. 1415-2500)
- Polypropylene storage box (USA Scientific, cat. no. 2310-5848)
- Erlenmeyer flask (Corning, cat. no. 4980-500)
- Glass pipette and bulb (Fisher Scientific, cat. nos. 13-678-20 and 03-448-25, respectively)
- Glass desiccator (Corning, cat. no. 3081-250)
- Vacuum grease (Dow Corning, cat. no. 1597418)
- Chemical fume hood
- Biosafety cabinet or laminar flow hood (e.g., Logic+ Class II A2 Biological Safety Cabinet, Labconco, cat. no. 302611100; or Console Horizontal Airflow Workstation, Nuair, cat. no. NU-301-530)

- Auto-desiccator cabinet (Bel-Art, cat. no. F42074-0116)

Seed preparation • TIMING 5 min

1. Pass dried Arabidopsis seeds harvested from healthy plants through two metal sieves with sieve mesh size 50 placed on top of sieve mesh size 60. Only collect the seeds in between two sieves. This will result in selection of seeds with sizes between 250 µm and 300 µm and reduce variation in germination rates.

PAUSE POINT Seeds can be stored under dry, cool conditions for at least one year prior to further processing.

Seed sterilization • TIMING 6-8 h

- 2 Aliquot approximately 50-250 seeds into a labeled 1.5 mL microcentrifuge tube. Do not close the lid. Repeat for the desired number of aliquots.

CRITICAL STEP Chlorine gas generated in subsequent steps will react with some commonly used inks and may interfere with sample labeling. Use chemical-resistant inks.

- 3 Place open microcentrifuge tubes in a plastic microcentrifuge storage box, but do not close the box lid. Place the open microcentrifuge storage box with open microcentrifuge tubes containing seeds and an Erlenmeyer flask containing 100 mL undiluted bleach in a glass desiccator located in a chemical fume hood.

- 4 Carefully add 1-2 mL of concentrated HCl using a glass pipette to the Erlenmeyer flask containing bleach and immediately place the lid on the glass desiccator, ensuring a proper seal. Sterilize seeds for 6-8 hrs³⁰.

! CAUTION Chlorine gas is toxic to humans! Use proper safety precautions.

! CRITICAL STEP Vacuum grease can help ensure a sufficient seal is made.

- 5 After sterilization, allow seed aliquots to off-gas residual chlorine gas before closing the lids on individual seed aliquots. Close the storage box lid and store the entire box containing seed aliquots at 4°C. For long-term cold storage, store seeds in the dark under low humidity. We found seeds stored in an auto-desiccator cabinet were sterile and viable after more than one year in storage.

! CAUTION Chlorine gas is caustic! Use proper safety precautions.

! CRITICAL STEP Residual chlorine gas can be removed by cracking the lid to the desiccator for several minutes and moving the seeds to a laminar flow hood. It is important to maintain sterile technique upon sterilization.

! CRITICAL STEP Checking for effective decontamination of an aliquot of seeds is crucial for maintaining axenic growth conditions (see Box 4).

! CRITICAL STEP Storing seeds in the dark at low humidity is important to maintain high germination rates.

PAUSE POINT Aliquots of seed can be sterilized in bulk and stored under appropriate conditions for future use.

Seed stratification • TIMING 2 d

- 6** Prior to an experiment, allow seeds to imbibe during a 48-hour stratification period in sterile Milli-Q water at 4°C in the dark prior to sowing. This helps promote uniform germination.

? TROUBLESHOOTING

Box 3 |**Plant growth conditions**

A plant tissue culture growth chamber (Percival) was used for growing plants in gnotobiotic setups. We routinely use the following conditions for Arabidopsis plant growth: 22 °C with 12h day/12h night photoperiod cycle at $\sim 90\text{-}100 \mu\text{E m}^{-2} \text{s}^{-1}$. The light intensity and temperatures on the tissue culture chamber were adjusted based on measurement done using probes placed inside the Microboxes to attain the expected growth parameters. We recommend rotating gnotobiotic boxes in a growth chamber every 2-3 days to ensure uniform plant growth. Since Microboxes are engineered to have a high water retention capacity inside the containers make sure to adapt Microbox-grown plants to desired relative humidity at the time of performing experiments, if relevant.

Box 4 |**Assessment of the sterility of gnotobiotic systems**

This box presents culture-based methods for testing the sterility of the gnotobiotic systems. To ensure axenic conditions are maintained throughout plant growth, it is important to check the sterility both before the experiment and after plant growth. For plants grown in the FlowPot system we test 7-10 days old seedlings at the time of thinning (Procedure1, Step 20) and again at the time of using plants for planned experiments. For plants grown in the GnotoPot system we check sterility of axenic plants and peat substrate at the time of using plants for planned experiments.

Reagents

- R2A agar medium (DIFCO, cat. no. 218263)
- Potato dextrose agar (PDA) (BD Difco, BD 213400)
- Standard sterile Petri plates, 100 x 15 mm (VWR, cat. no. 25384-302)
- Sterile tweezers or disposable inoculation loops (Fisher Scientific, cat no. 16-100-110 or 08-757-133, respectively)

REAGENT SETUP**R2A**

- Dissolve 18.2 g of powder in 1 L of water. Mix thoroughly. Autoclave at 121°C for 20 minutes on liquid cycle. Cool the medium to ~65°C and pour it into petri dishes in a sterile hood. Once solidified, the plates can be stored at 4°C for at least three months. R2A media from Difco contains: yeast extract (0.5 g/L), proteose peptone No.3 (0.5 g/L), casamino acids (0.5 g/L), dextrose (0.5 g/L), soluble starch (0.5 g/L), sodium pyruvate (0.3 g/L), dipotassium phosphate (0.3 g/L), magnesium sulfate (0.05 g/L), agar (15 g/L).

PDA

- Dissolve 39 g of powder in 1 L of water. Mix thoroughly. Autoclave at 121°C for 30 min on liquid cycle. Cool the medium to ~65°C and pour it into petri dishes in a sterile hood. Once solidified, the plates can be stored at 4°C for at least three months. PDA from Difco contains: potato starch (4 g/L), dextrose (20 g/L), agar (15 g/L).

Testing for culturable microbial contamination of seeds • Timing 2-7 days

1. Check for seed-borne contaminants and germination efficiency by incubating an aliquot of sterilized seeds on R2A agar at 22°C for at least one week.

? TROUBLESHOOTING

Testing for culturable microbial contamination of plants and substrate • Timing 2-7 days

- 2** To test for bacterial contamination after plant growth, transfer plant material or small amounts of peat substrate to R2A agar plates. Spread peat material evenly. Incubate for at least one week at 22°C looking for any possible bacterial contamination.
 - 3** Use the same approach and plate on PDA to test for fungal contamination. Incubate for at least one week at 22°C looking for any possible fungal contamination.
- ? TROUBLESHOOTING

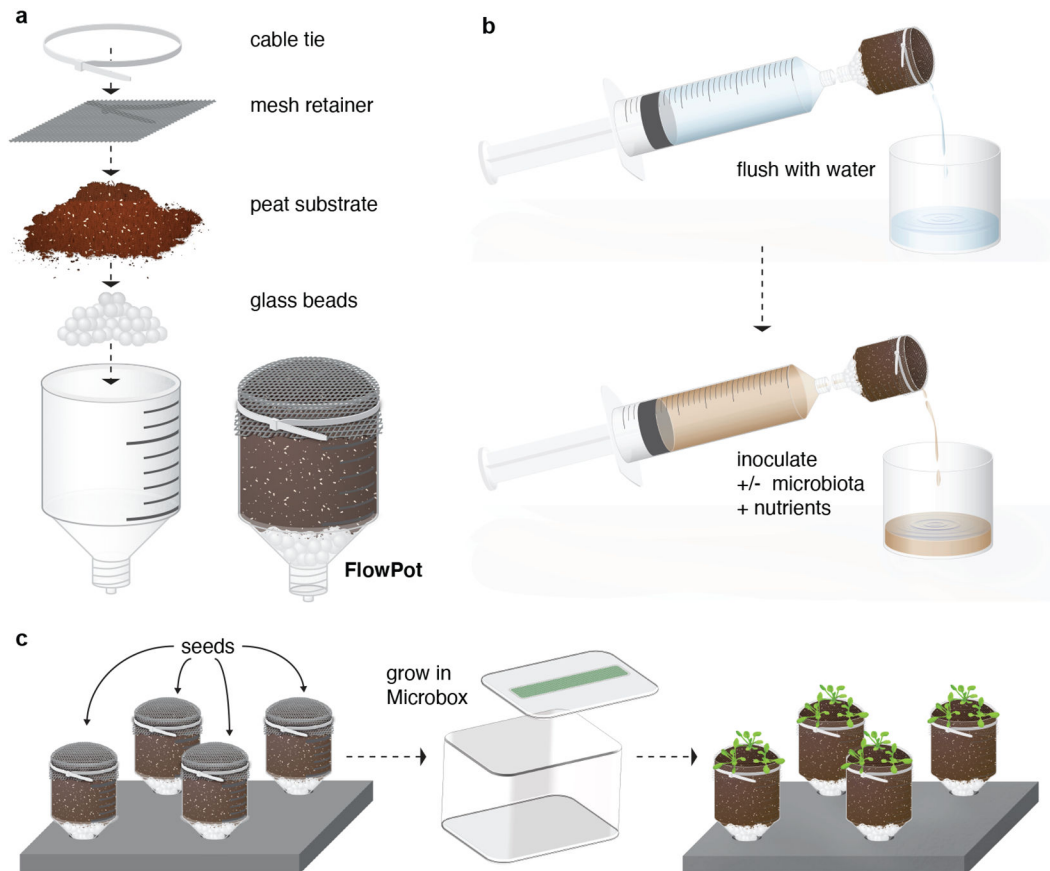


Fig. 1 | Schematic illustration of the FlowPot system.

Each FlowPot is prepared by **(a)** adding glass beads to the Luer end of a truncated syringe, followed by the addition of twice-autoclaved peat, covered with a mesh retainer, and then secured with a cable tie. Assembled FlowPots are then autoclaved, **(b)** aseptically irrigated with sterile Milli-Q water, and inoculated with nutrients and any desired input microbiota. **(c)** FlowPots are then aseptically placed into Microboxes on stands and microbe-free *Arabidopsis* seeds are sown onto each FlowPot. The Microboxes containing FlowPots are placed in a growth chamber with desired lighting and temperature conditions for plant growth.

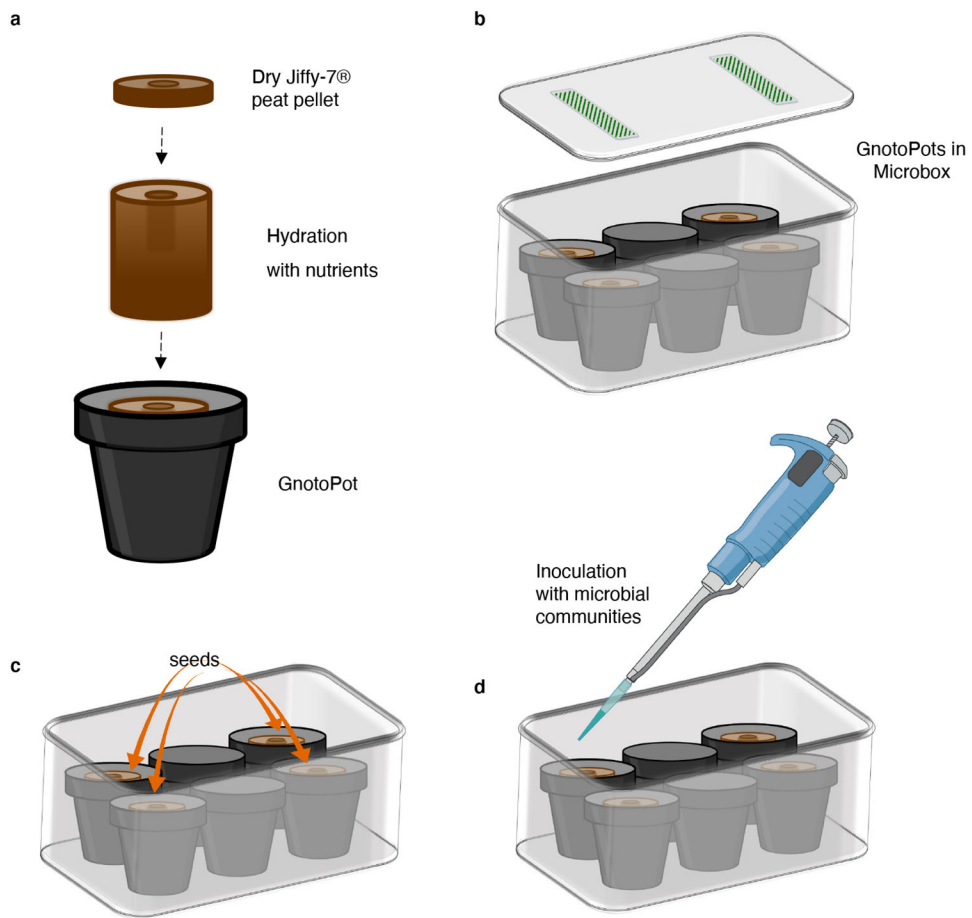


Fig. 2 | Schematic illustration of the GnotoPot system.

Soaking the Jiffy-7® peat pellet with nutrient solution results in expansion of the dry discs and formation of the GnotoPot (a). GnotoPots are placed inside the Microboxes flanking two empty pots (b). After the autoclaving cycles the GnotoPots are rehydrated with nutrient solution and seeds are sown aseptically (c). Lastly, desired input microbiota is inoculated using a 1 mL pipette (d), the lids are snapped closed and Microboxes are transferred to growth chambers.

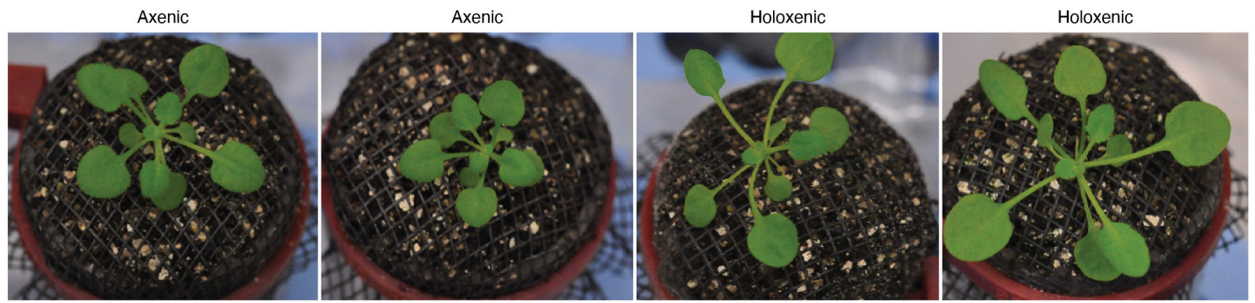


Fig. 3 1. *Arabidopsis thaliana* grown in FlowPots with axenic or holoxenic substrate. Growth phenotype of *Arabidopsis* plants grown in FlowPots, 4 weeks post germination. Holoxenic substrate was inoculated with a soil slurry, and axenic substrate was inoculated with a heat-killed version of the same soil slurry. Rosette images are representative of at least three replicated experiments.

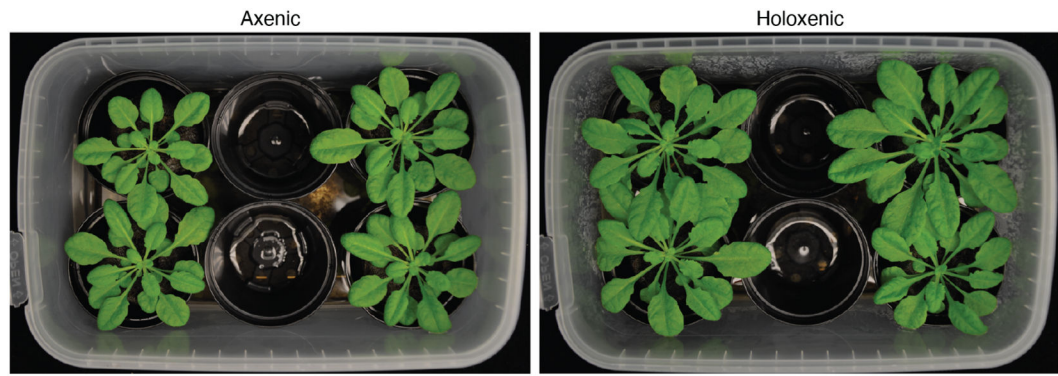


Fig. 4 | *Arabidopsis thaliana* grown in GnotoPots with axenic or holoxenic substrate. Growth phenotype of *Arabidopsis* plants grown in GnotoPots at 6.5 weeks post germination. Holoxenic substrate was inoculated with a soil slurry, axenic substrate was inoculated with a heat-killed version of the same soil slurry. Rosette images are representative of at least three replicated experiments.

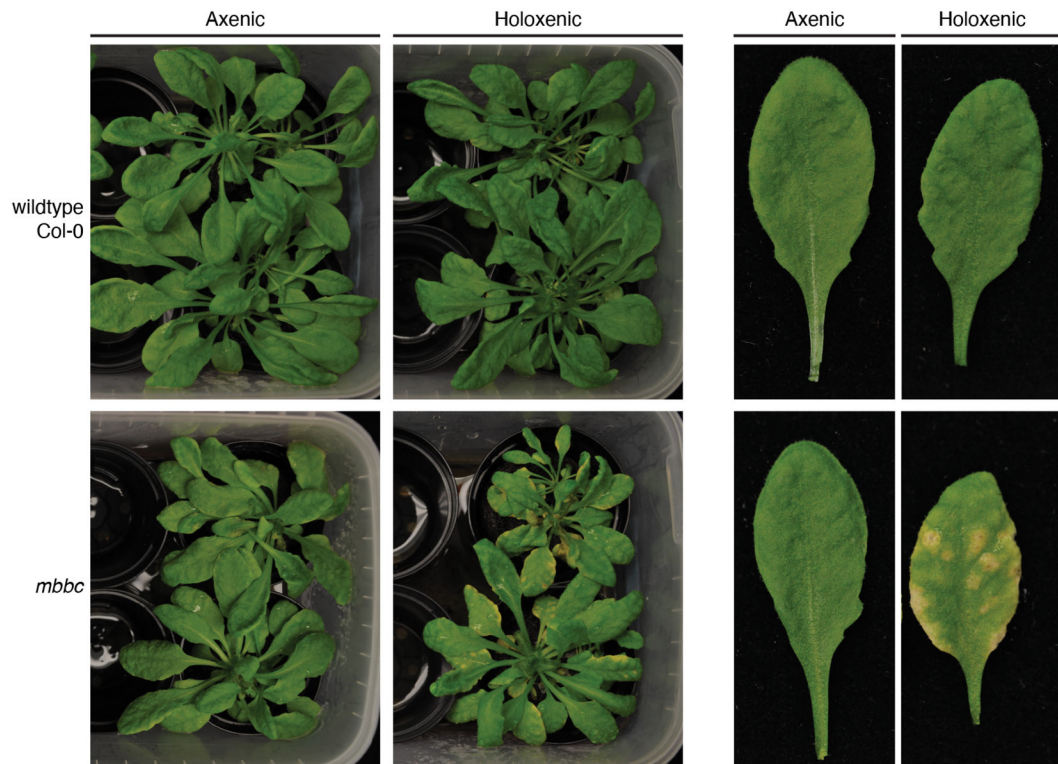


Fig. 5 |. Application for microbiota function studies *in planta*.

Six-week old wildtype (Col-0) and *mbbc* plants were subjected to high humidity (95% relative humidity) for 11 days. A chlorosis phenotype is observed in holoxenic *mbbc* plants but not axenic *mbbc* plants, indicating that chlorosis is dependent on exposure to a microbial community. Holoxenic substrate was inoculated with a soil slurry, axenic substrate was inoculated with a heat-killed version of the same soil slurry.



Fig. 6 I. Application for microbial community studies.

FlowPots were inoculated with soil slurries from three distinct environments (arid, undisturbed prairie, agricultural) (Supplementary Table 1) with 20 replicate FlowPots per treatment contained in 5 Microboxes (4 per box). Arabidopsis seeds (wildtype Col-0) were sowed, and total rosettes were collected at 4 weeks. Each sample consists of bulk DNA extracted from 4 pooled rosettes from the same Microbox. The heatmap represents the relative abundance of Operational Taxonomic Units (OTUs) for all OTUs that accounted for >2% of total reads in any given sample. Genus-level classifications are given for each OTU (Supplementary Table 2). See Supplementary Methods for DNA extraction and microbial community profiling.

Table 1.

Advantages and disadvantages of substrates used in gnotobiotic systems

Growth system	Advantage	Disadvantage
Nutrient agar	<ul style="list-style-type: none"> Chemically-defined substrate. Routine tissue culture methodology. 	<ul style="list-style-type: none"> Lacks the physical structure of soil for plants and microbes. Lack of organic matter relevant to soil. Non-uniform nutrient and O₂ delivery over time. Highly dissimilar to field condition.
Hydroponic	<ul style="list-style-type: none"> Chemically-defined substrate Easily accessible roots for exudate collection No substrate to interfere with imaging technology 	<ul style="list-style-type: none"> Lacks soil-like physical structure for plants and microbes. Lack of organic matter relevant to soil Highly dissimilar to field conditions Susceptible to contamination
Mineral substrates (calcined clay, sand, quartz, vermiculite)	<ul style="list-style-type: none"> Provides soil-like scaffold. Substrate highly accessible. Easy to sterilize. Roots are easily extracted from substrate. 	<ul style="list-style-type: none"> Lack of organic matter Lacks significant organic carbon, unless supplemented. Variable labile ions. Maybe difficult to maintain water flow
Sterilized Soil	<ul style="list-style-type: none"> Natural substrate for plant growth Easily accessible. 	<ul style="list-style-type: none"> Soil is a generic term that includes many diverse substrates with different edaphic features. Different soils are differentially impacted by autoclaving, and other sterilization methods. Not easy to standardize for a global research community. Roots not easily removed from substrate.
Peat (FlowPot and GnotoPot)	<ul style="list-style-type: none"> Peat is standard potting mix, and easily accessible around the world. Organic matter supports microbial growth and plant growth. Commonly used in commercial greenhouse operations. 	<ul style="list-style-type: none"> For the FlowPot system, assembly requires significant hands-on time Roots not easily removed from substrate.

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Table 2.

Troubleshooting table.

Procedure	Step	Problem	Possible reason	Solution		
Box 2	6	Poor germination rate	Sterilization is too harsh.	Reduce exposure to chlorine gas or reduce the amount of HCl added to the Erlenmeyer flask. Verify germination rate on 1/2X LS plates without sucrose.		
			Seeds storage suboptimal.	Ensure seeds are stored in the dark with low humidity. Verify germination rate on 1/2X LS plates without sucrose.		
Box 4	1	Seeds are contaminated	Seeds were not properly decontaminated.	Use fresh bleach during sterilization and ensure adequate sterilization time. Alternatively, increase the exposed surface area of seed aliquots by reducing the number of seeds in each tube. It may be necessary to empirically determine the duration of sterilization based on the volume of seeds in individual aliquots. Alternatively, surface sterilize seeds with Ethanol solution (50% Ethanol (v/v) +0.01% Triton X-100) for 5 min, followed by fresh bleach solution (10% commercial bleach+ 0.01% Triton X-100) for 5 min and wash 4 times with sterile Milli-Q water.		
			3	Plants or substrate are contaminated	Substrate was not properly sterilized.	Ensure sufficient time between autoclave cycles (24-48 h) during substrate sterilization. The rest provides an opportunity for dormant spores to germinate, which can then be killed during the second autoclave cycle.
					Tissue culture box not sealed.	Check filters and seals of Microbox. Discard if damaged.
1 - FlowPot	12	Microbox becomes deformed after autoclaving.	Water or media was contaminated during irrigation or inoculation.	Ensure clean, undamaged glassware and proper sterile technique are being used.		
			Lid was sealed on the Microbox while autoclaving.	Do not seal lids while autoclaving and follow manufacturer's instructions for Microbox sterilization.		
			15	Liquid is unable to be passed through the FlowPot.	Glass beads or debris are obstructing the irrigation port.	Insert a sterile needle into the bottom of the FlowPot to clear the irrigation port.
			15	The mesh retainer is dislodged from the FlowPot.	Glass beads or debris are partially obstructing the irrigation port, resulting in increased backpressure.	Insert a sterile needle into the bottom of the FlowPot to clear the irrigation port.
					Substrate is packed too tightly into the FlowPot, resulting in increased back pressure.	Assemble FlowPots using less substrate and do not pack.
					The nylon cable tie relaxed during autoclaving.	Do not fully tighten cable ties until after autoclaving or use a cable tie gun. Alternatively, change the material of the cable ties and avoid nylon.
			20	Plants appear chlorotic	Recalcitrant byproducts of sterilization may be deleterious to plant growth.	In our experience, using 50-60 °C water to flush the substrate improves performance if plants appear chlorotic.
20	Excess condensation builds up on the walls of the Microbox during plant growth.	Too much moisture is contained within the system.	The depth filter of the described Microbox is hydrophobic, so water is generally retained within a sealed box for quite some time. Decrease the amount of FlowPots per box to reduce excess moisture. We recommend no more than four FlowPots per Microbox.			
		Temperature fluctuations within the Microbox are increasing the rate of evaporation from FlowPots.	Maintain constant temperatures within the Microbox. Adjusting day/night temperatures can help account for the radiant energy absorbed by the Microboxes from the light source. Alternatively, we found that placing growth chamber temperature probes into sealed Microboxes with humidity levels corresponding to those of boxes growing plants helped mitigate temperature fluctuations. Additionally, insulating Microboxes from metal surfaces by placing			

Procedure	Step	Problem	Possible reason	Solution
				them on matte black ceramic tiles may also help reduce thermal fluctuations and the build up of condensation.
	20	Plants are water stressed.	Substrate is drying out.	Aseptically apply ~8 mL sterile water with a needle and syringe to the center of each FlowPot, avoiding damage to plant roots. We have found that watering plants after sowing is generally unnecessary and that the substrate sustains plant growth for at least 8 weeks without intervention. In our experience, the drying of substrate is usually due to temperature fluctuations increasing the rate of evaporation. The water is usually retained within the system on the bottom or sides of the Microbox as the filter is hydrophobic.
	20	Plants are nutrient stressed.	Nutrients are depleted from the substrate.	Apply ~8 mL 1/2X LS media with a needle and syringe to the center of each FlowPot as needed. We have found that supplementing FlowPots with nutrients after sowing is generally unnecessary and that the substrate sustains plant growth for at least 8 weeks without intervention.
	20	Plants fail to germinate.	Seeds sterilized for too long.	Decrease sterilization time. It may be necessary to empirically determine the duration of sterilization based on the volume of seeds in individual aliquots. We found that 6 h sterilization time had no adverse effect on germination rate. Alternatively, liquid bleach can be used to sterilize seeds.
2 - GnotoPot	2	Occasionally, the dry pellets do not fully expand.	Variance in manufacturing.	Visually inspect the dry pellets before placing inside the 2 inch pots to make sure that the mesh covering peat is not ruptured. Also after full expansion make sure that the central part of the Jiffy-7 [®] is accessible for placing the seed at the time of seed sowing. If the central hole is not accessible use a pair of tweezers to manually open up the mesh.
	26	Too much algal growth inside the box.	Excess of microbial community inoculum from soil.	Use a smaller amount of the soil for preparing the soil tea. Also make sure that the soil inoculum is only added to the central area of peat pellet
	26	The peat pellet looks dry after 6 weeks.	Excessive water loss	Although the GnotoPots perform very well in retaining the moisture, depending on the changes in the seasonal ambient humidity levels in different geographical locations some pots might have a drier peat pellet later in the growth phase. To hydrate these pots simply raise one side of the Microbox to about 10 degree angle to move the sitting solution to one side of the box and wait for 1-2 minutes. Perform this step for all the relevant controls as well.