Environments-by-Design: Developing new tools to bring "field conditions" to the laboratory

Kara Lind
Iowa State University
Environments-by-Design: Developing new tools to bring “field conditions” to the laboratory

by

Kara Lind

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Program of Study Committee:
Ludovico Cademartiri, Major Professor
  Martin Thuo
Rebecca Cademartiri
Patrick Schnable
William Beavis

The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this dissertation. The Graduate College will ensure this dissertation is globally accessible and will not permit alterations after a degree is conferred.

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DEDICATION

I dedicate this dissertation to my parents for providing me constant support and prayer, and Aaron, my brother, for always believing in me and encouraging me to pursue science.
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ABSTRACT

Maximizing agricultural yields is an urgent priority for our society as the world population will increase to 9 billion by 2050. Additionally, climate change could have detrimental effects on food supply and biomass production. Addressing these problems means considering the relationship the environment has on a plant’s development. The reality is though that there are few tools to plant scientists that can properly control and monitor the growing environment for plant. We believe that as engineers we can provide plant scientists with new tools which we call “environments by design”. These tools can control the environmental stimuli either abiotic (e.g. water availability, nutrient concentration, temperature, relative humidity) or biotic (e.g. microbes or other plants) delivered to plant in time and space. By controlling the environment, hypotheses of how genetic traits interact with environmental factors to yield the plant phenotypes can be addressed inside the laboratory.
CHAPTER 1. ENVIRONMENTS BY DESIGN

Research Motivation

Plants serve a fundamental role in society by being the basis of human civilization. Humans rely on plants for their food either directly or indirectly (e.g. feed for domestic animals). Additionally, plants can be harvested and used for industrial products (e.g. fuels, structural fibers) or medicine.

Increases in agronomic yields in the last century have been the result of agricultural practices like selective plant breeding [1, 2], genetically engineered crops [3], and application of agrochemicals and/or irrigation technology [4].

In this century, climate change and population growth represent major risks to global agronomic supply [5-7]. By 2050, the world population estimate is 9.8 billion people[8]. Feeding the world will be challenge with existing agricultural technology. Additionally, climate change could mean increasing temperatures which could result in shorter and more intense seasonal flows of river systems causing flooding [9] that could limit future crop production due to soil erosion [10]. Temperatures above 30°C will limit biomass of industrial crop varieties (i.e. corn, soybean) [11]. Overcoming environmental stresses could mean depletion of water or expansion of cropland, which could have implications on ecological biodiversity [12].

Computational modeling is one predominant tool used by agronomists to evaluate the potential effects climate change will have on our current crop varieties (i.e. biomass production) [13, 14]. While these approaches offer insight into they cannot overcome the challenges to increase productivity. Instead, the biggest effort is being conducted by genetically engineering or breeding crops with the hopes of finding the genes that regulate how plants can overcome current and future environmental stresses [5, 15]. One promising solution to help plants adapt to abiotic stresses, such as drought or heat stress, is to find stress-tolerant germplasm through experimentation in laboratory or greenhouse trials [16] and use plants with this genetic material in field trials [17, 18].
The main problem in conducting research on stresses is the lack of accurate models of environmental features[19]. Simplicity can be achieved when testing certain environmental features through use of such things as nutrient solutions in hydroponic or gels in growth chambers with controllable conditions like light intensity, temperature, and relative humidity. Additionally, plants can be isolated to allow simplification of phenotyping and data collection by the use of individual pots or other common growth devices (e.g. magenta boxes or petri dishes). While these simplifications can help facilitate research on genotype by environment interactions, they often create conditions that are not physiological (e.g. they confine the plant, they are sterile, they use growth media dissimilar than soil). This creates a gap between how phenotypes appear in the lab to those within the field [20].

Making better predictions of phenotypes could be addressed by the development of field-based phenotyping for roots and shoots however, these methods will still not allow us to test what can be or what might be. Instead, to troubleshoot real plant growth with new environmental loads we must be able to create highly controlled environments inside the lab.

So what is a highly controlled environment? In one case study, ten of the best laboratories that grow *Arabidopsis thaliana* in the world asked the question could they replicate phenotypes across laboratories under highly controlled conditions [21]? Detailed protocols were distributed along with identical pots, soil, and the same seed stock to each laboratory. Despite this, similar phenotypes were only present in four of the ten laboratories. Their conclusion that even small variations in growing conditions such as light quality and handling of plants can account for significant difference in phenotypes thus displaying the strong connection between a genotype by environment component in the lab [21].

Stringent protocols, robotic handling, and other customizations might increase reproducibly within and between laboratories but that does not mean the environment is highly controlled. To be a highly controlled environment, any flow within the system must be able to be set or manipulated or at the very least monitored and recorded. Evaluating
current environments used in plants science, we and others find most are not highly controlled[22]. In fact, one of the most heavily used environments, the Petri dish (>16K papers use for plant protocols according to Google scholar) does not consider the control of the atmospheric environment. By sealing the Petri dish, sterility is retained at the expense of the gas exchange in system. Without this ventilation, plant phenotypes differ from plants grown in ventilated environment [23]. If we continue to use the Petri dish as an environment, we must consider if the hypothesis we are trying to test is compatible with the limitations of the device.

Plants are sessile organisms that are conditioned to grow according to environmental cues. A plants response to environmental cues is a tropism. Significant work has been devoted to the study of tropisms, namely hydrotropism. Knight first characterized hydrotropism in 1811 as the phenomena that enables roots to respond to moisture gradients by bending towards areas of higher water availability [24]. Determination of the mechanism of hydrotropism could be valuable for our understanding of plant regulation and water resource utilization [25]. Understanding and determining the mechanism of hydrotropism has been difficult as it is challenging to design well-controlled experiments to study it and the tools to study roots under conditions of water stress are limited [26]. Current approaches rely on establishing water potential gradients by introduction of osmoticums into gels. Osmoticums are chemical agents like glycol [27] or sorbitol [28], which can create asymmetric water deficits in the plant tissues. Other approaches create water gradients by creating humidity gradients in air in closed chambers [29] or rely more on the production of drought stress by depletion of water. While these approaches are useful they do not properly control for other tropisms (like gravitropism and phototropism), both of which could over-power the hydrotropic response. In fact, to our knowledge, there is not a single highly controlled growth environment or tool that enables the generation of arbitrary and stationary distribution of water for the study of plant hydrotropism. At the molecular and root system level, the study of hydrotropism will require new experimental platforms that we believe as engineers we can help develop.
Our Approach

We believe that there are significant opportunities for engineers to contribute to the field of plant science through the creation of new technologies that will enable more data collection as well as hypothesis based experimentation. In the last decade, the majority of engineering involvement in plant science has been focused on the creation of new high-throughput phenotyping technologies for the field [30] or greenhouses [16]. To make use of these technologies, hundreds of plants must be analyzed per day, data must be collected, stored and analyzed. Machine learning can then be used to find correlations between the environment and plant phenotype [31]. This approach to establishing genotype by environment is extremely valuable and could very well lead to new breeding programs.

Field based phenotyping approaches have the advantage of being able collect data on fully grown plants that are economically useful under natural environmental conditions [32]. Field based approaches however, have some limitations, (i) they are constrained to seasonal growing conditions and (ii) they focus almost exclusively on shoot phenotypes. High-throughput devices for greenhouses and growth chambers have the advantage that they can be used all year long thus accommodating more experimental cycling but still often do not accommodate root phenotyping. One limitation of these devices is that individual plants cannot be controlled for specific environmental factors like temperature or relative humidity. It is our belief that plant science could benefit from the creation of individual plant chambers that allow the research to input or control the specific environment for that plant. We call these types of tools “environments by design”.

These environments have been engineered to control a single plant’s abiotic environment (e.g. nutrient concentrations, temperature, relative humidity, water availability, aeration) and biotic interactions (e.g. microbes, other plants). From an engineering standpoint, using individual plant chambers has many advantages. Controlling some environmental parameters (e.g. relative humidity and sterility) is more logistically and economically difficult in large growth chambers. Single plant environments allow for hypothesis driven experimentation where plants can be individually stimulated by a wide range of stimuli. Individual and accurate control of the environments within these could
reduce replicate numbers for a given experiment as well as improve reproducibility within the laboratory. Improving rates of reproducibility while simultaneously reducing replicate numbers could ultimately make phenotypic predictions quicker.

We found that there are an array of parts available in the marketplace for purchase that can be assembled together to create “environments by design”. Silicone polymers and other plastic injected pieces (i.e. polycarbonate LEGO® bricks) are cheap and can be assembled to create environments that are scaled for studying plants while still maintaining chemical inertness and autoclave capabilities needed for biological experimentation. We believe the tools can be assembled and operated by plant scientists as they do not require extensive fabrication or training to use as compared to other approaches like microfluidics [33]. Other components like Arduino based sensors and pumps can be integrated into these environments to be able to test more sophisticated hypotheses like the response of plants to biotic stimuli[34]. Additionally, the creation of wax paper microfluidics using a cheap Xerox printer can create scaffolds to study important phenomena such as root responses to water distributions (i.e. hydrotropism) while still simultaneously being able to root phenotype [35].

**Dissertation Organization**

The remaining chapters of this report will address the tools we have generated that enable simple hypothesis driven research. Chapter 2-4 showcase scalable and modular 3-dimensional growth environments. LEGO brick environments in chapter 2 can be used for hypothesis testing such as how roots respond to gradients of nutrients. Our HOMES (Habitats for Organisms and Modular Ecosystems) can be used for more sophisticated testing such as how plants respond to biotic stimuli. Chapters 5 and 6 detail 2-dimensional tools for studying root development with controlled relative humidity. Chapter 7 details the use wax paper printing as a means to study water stress and hydrotropism of model plants on the lab benchtop.
References


CHAPTER 2. LEGO® BRICKS AS BUILDING BLOCKS FOR CENTIMETER-SCALE BIOLOGICAL ENVIRONMENTS: THE CASE OF PLANTS

Modified from a paper published in PLOS One

Kara R. Lind†, Tom Sizmur‡, Saida Benomar‡, Anthony Miller†, Ludovico Cademartiri‡,*

† Department of Materials Science & Engineering, Iowa State University, Ames, IA, 50011
‡ Ames Laboratory, US Department of Energy, Iowa State University, Ames, IA, 50011
‖ Department of Agronomy, Iowa State University, Ames, IA, 50011
△ Department of Chemical & Biological Engineering, Iowa State University, Ames, IA, 50011

Abstract

LEGO® bricks are commercially available interlocking pieces of plastic that are conventionally used as toys. We describe their use to build engineered environments for cm-scale biological systems, in particular plant roots. Specifically, we take advantage of the unique modularity of these building blocks to create inexpensive, transparent, reconfigurable, and highly scalable environments for plant growth in which structural obstacles and chemical gradients can be precisely engineered to mimic soil.

Introduction

Microfluidics[1], and other engineered environments[5], [6] can produce highly controlled micrometer-scale environments for the study of organismal model systems (e.g., mammalian cells). However, scientists or engineers interested in manipulating the environment of cm-scale organisms (e.g., plants) have remarkably few convenient tools at their disposal[7], [8]. This paucity is partly due to the demanding design requirements associated with larger scales (e.g., cost). This liability is particularly evident in the study of plants and their root systems.

The development of plants in soil is an important subject of investigation. The provision of food to the global human population is under severe pressure (our supply of food is predicted to be far below demand by 2050[9]) and depends on plant roots[10] (97.6% of global calorie consumption is derived from plants[11]). Roots influence a plant's yield and whether a plant will survive stresses. We know that root growth is
strongly affected by its environment, soil, but our mechanistic understanding of these effects is imperfect[10], [12] and strongly limited by technical challenges.

Root development is a difficult process to study experimentally. (i) Plants display highly variable root systems, even when genetically identical[13]. (ii) Roots are remarkably sensitive to a variety of stimuli (e.g., gravity, light, touch, moisture, nutrients, oxygen, temperature, trauma, electric fields[14]). (iii) Any volume of soil is unique and impossible to replicate exactly[15], [16]. (iv) Its heterogeneity makes it opaque to most forms of radiation[17]. (v) Its structural and chemical characteristics (i.e., porosity, surface chemistry, nutrient gradients, oxygen gradients, bulk composition, soil biota) cannot be independently manipulated.

One approach to avoid this complexity is to characterize the growth of plants in soil-less media, e.g., hydrogels, paper, glass beads, sand. These systems are less inhomogeneous and irreproducible than soil and can be modified – usually to a limited extent – to mimic soil properties such as chemical composition [18], physical structure [19], [20], water availability [21], refractive index [22], or mechanical strength [23]. However, the lack of modularity, versatility, structural precision, and the very limited control over structural and chemical heterogeneities in these systems severely limits the type, complexity, and reproducibility of the experiments they can perform. Microfluidic approaches offer fascinating capabilities for the study of plant roots, but are subjected to limitations in their throughput and in the size of the plants they can host [4], [24], [25].

We here demonstrate that LEGO bricks are highly convenient and versatile building blocks for building cm-scale engineered environments for plant roots. Their modularity enables the fabrication of environments with highly controlled structural and chemical heterogeneities that are suitable for convenient quantitative studies of environmental effects on plant phenotypes[26].

**System Design**

A convenient experimental platform for the study of root development in controlled environments must satisfy a demanding set of design constraints. LEGO bricks, while conceived and sold as toys, satisfy these constraints.
Modularity. Modular systems can produce many structurally distinct environments from a few different components. Features can be added or removed without remanufacturing the entire experimental setup. LEGO structures are modular. The smallest bricks are 8x8x6 mm. The largest are 48x8x50 mm. The number of different structures that can be made with these units is staggering: six identical bricks can form almost a billion different structures[27].

Scalability. Confinement can affect the physiology of an organism[28]. The ability to create experimental platforms of a range of sizes enables researchers to study any plant and their ensembles. LEGO structures can be easily scaled to accommodate different plant species: the smallest enclosed environment that can be produced with LEGO bricks measures 0.35 cm³ in volume, and it is theoretically possible to create LEGO structures capable of containing the largest plant species.

Structurally precise. Roots are sensitive to the physical structure of their environment. For example, the study of root thigmotropism (the response of a root to touch) requires structures that are of an exact size and shape. The molds used to produce LEGO bricks are accurate to within 5 µm[29], which is comparable to the diameter of a root hair and to the resolution of 3D printing (minimum layer thickness is ∼50 µm in some of the best current models).

Capable of increasing levels of complexity. A good model system allows for the controlled introduction of experimental variables. LEGO bricks can be used – as shown below – for the generation of physical barriers, air pockets, chemical gradients, and interconnecting chambers to control the growth environment of a plant.

Simplicity. Simple setups reduce the risk of operator-induced systematic errors. Differently from microfluidic approaches, the assembly of structures from LEGO bricks does not require technical training so undergraduate students can perform LEGO brick-based plant experiments from their first day in the laboratory. Simple experiments that demonstrate fundamental principles of plant growth (e.g., tropisms) or encourage experimental creativity can be conducted by school children of all ages during science education classes[30].
Reproducibility. Plant root experimental platforms (e.g. sand columns, rhizotrons, split-root pots) are typically made from scratch. Their reproducibility between labs or across continents cannot be guaranteed. The unique selling point of LEGO bricks is that bricks bought in separate batches are essentially identical and backward- and forward-compatible with each other. Experiments created from LEGO bricks can be accurately replicated anywhere in the world.

Affordability. The more expensive each experiment is, the fewer experiments can be conducted with finite resources. This fact is especially meaningful in developing nations[31] and in research fields, like plant science, where throughput is an essential parameter. Individual LEGO bricks cost between $0.10 and $1.00 and are sold worldwide. A LEGO structure capable of growing a plant costs $3.1 and is reusable: some LEGO bricks in our lab have been in near-constant use for two years.

High throughput. The ability to run a large number of experiments at the same time is essential for the establishment, for example, of genotype-environment-phenotype relationships[32]. A LEGO structure like the one shown in Figure 1 can be assembled in less than a minute.

Transparency. Twenty eight different LEGO bricks are made from transparent polycarbonate which can be assembled into transparent structures for the real-time monitoring of plant roots over time.

Autoclavable. Tissue cultures require sterile conditions. Transparent LEGO bricks (with the exception of large base plates) are autoclavable due to their polycarbonate composition: they still fit together in the same way as they do prior to autoclaving and are still transparent after more than 50 autoclave cycles. Opaque LEGO bricks are made from acrylonitrile-butadiene-styrene block copolymer (ABS), and can be sterilized with ethanol or bleach.

Three-dimensionality. While 2D platforms offer significant advantages in terms of visualization and practicality[33], 3D mediums are more representative of the natural environment of roots[34]. LEGO bricks allow for the creation of nearly arbitrary 3D structures.
**Chemical inertness.** Legislative standards ensure the safety to children of LEGO bricks sold in the USA and EU. These standards include maximum soluble levels of toxic or hazardous substances.

![Diagram of the process of carrying out a plant growth experiment using LEGO bricks as building blocks.](image)

**Figure 1.** Scheme of the process of carrying out a plant growth experiment using LEGO bricks as building blocks. The same process can be used to prototype and fabricate other biological experiments.

**Compatibility with existing growth environments.** Tools that integrate with existing experimental platforms are often the most useful. The modularity of LEGO structures
enables them to integrate with laboratory protocols e.g., LEGO structures can hold gel, beads, sand, soil, 3D-printed elements, or be structurally precise elements in other setups[35].

**Results and Discussion**

Figure 1 shows a flow diagram of the design, assembly, disassembly, and re-assembly of an experiment based on LEGO® bricks. The website of the LEGO Group ([www.lego.com](http://www.lego.com)) provides a free software (LEGO Digital Designer, LDD) for the CAD-like design of structures using any available LEGO® brick. The software outputs a step-by-step assembly guide and a list of the required parts. Individual bricks can be purchased through the “Pick a Brick” section of www.lego.com or other outlets (e.g. local LEGO stores, EBay). Sterilization of LEGO® bricks can be performed before or after assembly. The preservation of sterility requires the structure to be maintained in a sterile container during the course of an experiment.

The simplest example of a plant germination and growth environment based on LEGO bricks is shown in Figure 1. The LEGO bricks are assembled into a container that contains a root growth medium on which a seed is germinated and grown: Figure 1, for example, shows a *Brassica rapa*, Wisconsin Fast Plant, Astroplant, *dwf1*, growing on a transparent hydrogel, Gellan gum. While gel media for root growth are very commonly used in experiments[26], they are not the best mimic of soil: root architectures grown in an homogeneous media will not match those of plants grown in real soil[36]. However, gel media allows us to demonstrate three essential capabilities of LEGO-based biological environments: their ability to hold liquids, their compatibility with real-time observation and root structure analysis, and their use in generating reconfigurable environments that include controlled heterogeneities. Furthermore, LEGO environments are not limited to gel media: the environment shown in Figure 1 can hold other media of choice, e.g., sand, perlite, soil.
Since structures built from LEGO® bricks are not waterproof, their use to hold gels requires some stratagems (see Supporting Information for details and Movie S1 for a demonstration). The LEGO structure must be chilled in a freezer before the cool gel solution is poured in it just prior to setting. Using this approach, leakage of the gel solution was minimal. These basic environments can be easily scaled to match the dimensions of the organism under consideration and the time the organism is allowed to grow. Figures 2a, 2b, and 2c show the use of LEGO® bricks to create containers with very different dimensions (5×5×5 cm, 10×10×5 cm, and 20×20×10 cm) for the growth of Fast Plants, *Triticum polonicum* (Wheat), and *Zea mays* (Corn).

Figure 2. Versatility, transparency, and modularity of the LEGO-based environments for plant growth. a-c) pictures of basic LEGO-based environments growing Fast Plants, Wheat and Corn. The size of the environments can be controlled to match the size of the organism under consideration. d) Timelapse imaging of *Lepidium sativum* root development through the walls of a LEGO-based environment. The images indicate the time since germination. e) Examples of a LEGO-based system that allows for the dynamic change of the environment of a plant. Two plants (Fast Plants) are grown in isolated environments. The environment is then modified, during growth, to allow the two plants to share the same environment and interact.
The transparency and flat walls of LEGO bricks allows for good quality real time imaging of the development of the root system. Figure 2d shows time-lapse imaging of *Lepidium sativum* (Garden cress) roots over the course of ~48 hrs from germination in a LEGO-based environment. The plant was chosen for its relatively fine roots (~350 µm thickness) that would have been hard to image in a poorly transparent system.

The reversible nature of the mechanical bond between the bricks provides two important capabilities: the creation of reconfigurable biological environments, and of highly controlled heterogeneities (i.e., solid obstacles, air pockets, and chemical and soil biota gradients) in an otherwise homogeneous growth medium. Figure 2e demonstrates a reconfigurable plant growth environment. Two Fast Plants were grown in gel in separate containers assembled on the same base plate. The LEGO brick walls separating the two containers were removed and reconfigured to make one larger container. The volume separating the two plants was then filled with more gel, fluidically connecting the two plants. Figure 3 demonstrates the generation of controlled heterogeneities in a homogeneous gel medium for plant growth by a simple templating strategy borrowed from the materials science “toolbox”. A gelling mixture was poured into a LEGO-based mold. LEGO-based features in the mold can be used as solid heterogeneities to study the physical interaction of plant roots with solid objects (thigmotropism). After gelation, LEGO-based molds could be removed, leaving behind precisely positioned air pockets that would serve as sources of oxygen gradients into the gel. These pockets could be then refilled with a hydrogel containing a desired chemical to generate precisely positioned one-dimensional (Figure 3, bottom left panel) or two-dimensional (Figure 3, bottom right panel) nutrient gradients. The above process can be combined to create environments with solid heterogeneities, air pockets (i.e., oxygen gradients), and chemical (e.g., nutrients, toxins, signaling molecules) gradients simultaneously (see Appendices S1).
Figure 3. Fabrication of controlled heterogeneities in plant growth environments. Sequence of diagrams and corresponding images illustrating the generation of a 1D and 2D heterogeneities (solid features, air pockets, and chemical gradients) across a developing root system of a Fast Plant. In the bottom panels, the red linear gradient is of MS nutrients (dye is added for visibility), while the radial gradients are from potassium phosphate (green), potassium nitrate (yellow), calcium chloride (red), and magnesium sulfate (blue).
Conclusions

In summary, we demonstrated that LEGO-based environments can (i) scale to the size of the organism under consideration, (ii) allow for real time monitoring of root systems in 3D, (iii) be structurally reconfigured to change the environment of an organism during its development, and (iv) generate precisely controlled heterogeneities (i.e., solid barriers, air pockets, chemical and soil biota gradients) in an otherwise homogeneous growing medium.

This manuscript also proposes a broader concept: the use of reusable and mechanically interlocking building blocks for the construction of biological environments for cm-scale organisms and systems of organisms. Modular and reusable building blocks can alleviate the challenges associated with the large scales of plant science experiments, while providing new capabilities (e.g., controlled heterogeneities, reconfigurable environments) for the study of environmental effects on biosystem development. Furthermore, this concept provides materials chemists and engineers with two stimulating opportunities: (i) to creatively engage with the synthesis or development of increasingly capable cm-scale biological environments for important organisms such as plants, and (ii) to use these environments to test hypothesis concerning plants that are compatible with their skillset. Compelling opportunities lie in extending our approach to chemically synthesized bricks, LEGO-compatible 3D-printed bricks and objects, and commercial bricks from other manufacturers. Our laboratory will be introducing a set of integrated tools for the fabrication of frugal but sophisticated cm-scale environments for the study of plants and other organisms.[37]

Materials and Methods

Limitations and Open Questions

While LEGO offers a remarkable set of assets for the design of environments for the growth of cm-scale organisms and systems of organisms, it does have limitations. Some of these limitations are intrinsic, while others can be plausibly overcome with more design work or by complementing this LEGO-based approach with other techniques.
We here review the limitations of our approach.

1. The approach is not exactly boundless in that it is bound by the supply of pieces that LEGO provides, over which the scientist has no control over. The general dimensions and backward compatibility of LEGO bricks will be most likely preserved. It is not guaranteed, however, that all currently produced transparent bricks will be always in production. We do not believe this to be a major concern. The number of structures that can be produced with minimal subsets of the existing catalogue is staggering. Furthermore, any such concern is strongly alleviated by the progress in 3D printing. Additive manufacturing could be easily used to compensate for the limitations in the choices of LEGO bricks.

2. The geometry of the LEGO system is based on right angles.

3. The delivery of individual pieces can take as long as three months, when ordering from the United States. As we mentioned before, other options (e.g., Ebay) exist that provide much faster delivery.

4. As we mentioned before, while a large number of LEGO bricks are autoclavable, there is a much larger range of brick types that are colored, composed of ABS plastic and thereby not autoclavable. Those bricks can be used for biological experiments, provided that they are sterilized by ethanol and/or bleach.

5. Producing a LEGO structure, such as a box, does not necessarily result in a liquid tight design. In the case of the gels, some leakage did occur during the experiments. Methods such as superglueing the bricks together could be done but result in a loss in future mobility of those bricks.

**Failed Experiments**

1. Acetone is not an effective method to reverse superglued LEGO bricks. 2. Wrapping cling wrap around the outside of the LEGO brick environment did not ultimately reduce gel leakage. 3. Dipping the entire LEGO brick environment mold in liquid gel did not close cracks and did not reduce gel leakage. 4. Gel concentrations below 3 g/l of Phytagel did not result in air-filled columns that remained intact for more than a day. 5. In a few experiments, the plant did not penetrate the gel and instead grew only on the surface of
the gel. This can often be prevented if the seed is slightly submerged in the gel when planted or gel is used to encapsulate the seed. 6. In a few experiments, the root was capable of going between the LEGO brick cracks and thus finding its way outside the LEGO environment.

References


CHAPTER 3. MILLIFLUDIC, MODULAR, “PETRI DISHES” FOR THE STUDY OF ORGANISMAL INTERACTIONS

Modified from a paper submitted

Oskar Siemianowski†, Kara R. Lind†, Xinchun Tian, Matt Cain‡, Songzhe Xu§, Baskar Ganapathysubramanian§, Ludovico Cademartiri†,2,3*

1 Department of Materials Science & Engineering, Iowa State University of Science and Technology, 2220 Hoover Hall, Ames, IA, 50011
2 Department of Chemical & Biological Engineering, Iowa State University of Science and Technology, Sweeney Hall, Ames, IA, 50011
3 Ames Laboratory, U.S. Department of Energy, Ames, IA, 50011
4 Department of Mechanical Engineering, Iowa State University of Science and Technology, Ames, Iowa, 50011
† These authors contributed equally to this work.

Abstract

Understanding the chemical signaling that underpins important ecosystems such as the rhizosphere and microbiomes requires quantitative knowledge of the concentrations of signaling molecules in time and space. This goal is a significant challenge, because the concentration of signaling molecules depend on the distance between organisms (often smaller than 2 mm due to diffusion limitations), as well as their size (from microns to centimeters) and growth stage. Detangling this problem requires control over mass transport that is beyond the capabilities of any of the commonly used growth environments (e.g., Petri dishes). For example, the slow rate of molecular diffusion in liquids complicates significantly the study of plant-plant and plant microbe interactions: for interactions to occur, the organisms must be separated by few hundreds of microns, which is especially challenging with cm-scale organisms such as plants.

We describe here a simple approach to create small model ecosystems as millifluidic networks of interconnected habitats (hosting microbes or plants), which offers (i) quantitative and dynamic control over the exchange of chemicals between habitats, and (ii) independent control over their environment. Oscillatory laminar flows produce regions of vortex mixing around obstacles. When these overlap, rapid mass transport by dispersion occurs, which is quantitatively describable as diffusion, but is directional and tunable in rate
over 3 orders of magnitude. We show that his acceleration in diffusion rate is quantitatively equivalent to reducing the distance between the habitats down to the length scales characteristic of signaling in soil (<2 mm).

**Introduction**

We are interested in understanding the collective behavior of communities of organisms, especially plants. Quantifying and comparing phenotypic responses to signaling is difficult, in part, because the common growth environments do not control mass transport (and therefore signaling) between organisms and cannot usually control their environments independently. In this paper we describe an experimental platform for the simple creation of networks of organisms (from plants to microbes) that offer (i) complete control over the exchange of chemicals between each organism, and (ii) independent and precise control over the environment of each organism.

The establishment of cause/effect relationships in the interactions between organisms is necessary to understand, protect, utilize, and predict the behavior of ecosystems[27-33]. Interactions between organisms either require contact (e.g., endophytes), or can occur at a distance[34, 35] by chemical signaling through a gas (e.g., pheromone signaling[36]) or a liquid phase (e.g., rhizosphere, quorum sensing, biofilm formation)[34, 35, 37, 38].

Petri dishes, Magenta® boxes, Falcon® tubes, well plates and other common growth environments were not designed to study organismal interactions[32, 39]: (i) their size is mostly fixed (limiting plant work to seedlings and co-cultures to few organisms)[27, 40, 41]; (ii) co-cultures must share the same resources and the same, poorly controlled, non-physiological environment (complicating the distinction between competition, environmental, and signaling effects[39, 42]); (iii) they provide limited control on what is being exchanged, at what rate, between which organisms, and by what part of each organism (complicating the identification of the signaling mechanism [43]); (iv) it is difficult or impossible to add, remove, or confine organisms or chemicals in them (limiting our
control on the evolution of microbiomes[27, 44]). These limitations are usually accepted due to the unavailability of practical alternatives, or perceived necessity of contact or close proximity (<1 mm) between organisms due to the slow rates of diffusion in liquids[40].

Microfluidic approaches[26, 45, 46] and other custom-designed tools[47, 48] allow for controlled μm-scale separation between organisms, but scale only with difficulty[49, 50] to larger organisms like plants, are time consuming, costly, and require engineering expertise and facilities that are usually not conveniently available in a biology laboratory.

**System Design**

Our simplicity-driven approach[51, 52] is based on stackable, customizable, cm-scale habitats (Habitats for Organisms and Modular Ecosystems, HOMEs) connected in fluidic circuits (“communities”). The basic unit – the 111 HOME (Fig. 1a) – is sterilized and sealed in one step by lining the gaps between components with Parafilm®, clamping them, and autoclaving at 121°C. The molten Parafilm® seals the gaps of the structure without gluing it together. Therefore, HOMEs are watertight (Fig S18), and nearly airtight (Fig S19), and can be autoclaved at least six times, after which they can be disassembled, cleaned, and reassembled in any chosen architecture. The design presented here can be assembled in under 5 minutes and uses commercially available components (i.e. LEGO® bricks for windows) and polycarbonate or glass sheets for roof and floor, which are sealed to the windows through a gasket of silicone foam. The physical environment in a HOME is easy to control with pumps (e.g. the CO₂ concentration can be set within 7±8ppm of an input gas, cf. Fig. S22) and heating elements powered by Arduino controllers (from lab temperature to 44.8±0.3 °C, Fig. S23).

While the 111 HOME is suited to the incubation of microbes, stacking two of them together, yields habitats for plant growth (112 HOMEs, Fig. 1A). The diaphragm separating the two units holds a gel-filled pipette tip in which the seed germinates, allowing the roots and shoot to grow in two independently controllable “rooms” of the same HOME.
Figure 1. Design and connectivity of HOMEs. (A) Schematic of the 111 and 112 HOME. (B) A *P. fluorescens* culture in a 111 HOME. (C) A *B. rapa* plant growing in a 112 HOME. (D-G) Different types of semipermeable connections controlling mass transport between HOMEs. (H) A HOME community featuring different types and sized of organisms connected through 0.2 μm filters, from the left to the right *P. fluorescens, B. rapa, T. polonicum* and *Z. mays*.
HOMEs can easily connect into “communities” to establish interactions between organisms. Pinching flexible silicone tubing between a hole in the windows and a rigid cylinder lodged inside the tubing creates couplings that are watertight, autoclavable, and reversible.

112 HOMEs can be connected in the air phase, above “ground”, to study volatile-based signaling[36] or in the liquid phase, below “ground”, to study non-volatile based signaling. Semipermeable membranes installed in the tubing control what is shared between HOMEs. Filters (0.2/0.8 µm mesh size) prevent bacterial migration and root propagation, but allow molecular and ionic transport. Coarser meshes (100 µm) confine the roots of small seedlings (e.g. Brassica rapa), but not microbes or chemicals. “Liquid” membranes consisting of perfluorodecalin droplets confine microorganisms and molecules, but not roots[53-55] (Fig. 1g).

HOME communities can address interactions that require contact or that can occur at a distance. For example, Figure 2a-b shows the selective colonization of plants by flowing an inoculum (Pseudomonas fluorescens) through a path delimited by valves in a HOME community (3x3 B. rapa). After a temporary exposure, the colonized HOMEs can be flushed with sterile media to remove unbound microbes, and the valves can be reopened and substituted with 0.2 µm filters to reestablish connections between colonized (Figure 2c) and non-colonized (Figure 2d) plants. The same approach can be used to selectively and temporarily target organisms with abiotic stimuli such as a toxins (Figures 2e-f, S24).

Interactions at a distance in liquids are usually limited by diffusion. In the absence of advection, the diffusion of molecules across cm-scale distances is extremely slow (148 days, assuming D=3.9·10^{-10} m²/s and a distance of 10 cm, see Fig. S27). Diffusion can be accelerated by shear, i.e., dispersion[56, 57]. By leveraging vortex mixing at the connections and using oscillatory flows, we amplified the effect of dispersion and combined it with zero net advection. Three 111 HOMEs were connected in series (Fig. 3a), separated by filters.
Figure. 2 Selective and Temporary Colonization and Stimulation. (A-B) 5 HOMEs within a 3x3 HOME community of *B. rapa* plants were selectively colonized by flowing *P. fluorescens* through a circuit delimited by valves. After 6hrs incubation, the colonized HOMEs were flushed with new sterile media. Syringe filters are used to prevent the colonization of sterile HOMEs while allowing molecular signaling to occur. (C-D) Comparison of colonized and non-colonized root systems 3 days after exposure. (E-F) 5 HOMEs within a 3x3 HOME community of *B. rapa* plants were selectively stimulated by flowing a solution of the toxin cadmium through a circuit delimited by valves. After 24 hrs incubation, plants within the selected pathway were flushed with Cd-free plant media and the connections between HOMEs were restored. (G-H) Comparison of stimulated vs non-stimulated plants (see SM for statistical analysis).
The HOME in the middle – the source – contained an aqueous solution of dye, while the others – the sinks – contained DI water and were attached to the same peristaltic pump. Inputting a square wave voltage function (4s period) in the pump resulted in an oscillatory motion of the fluid column (max. displacement = 0.4 cm and max. velocity = 0.4 cm/s, flow rate = 0.13 cm³/s, Re = 16)(Fig. 3b).

The oscillations caused the dye to transport outward from the source within hours instead of months (Fig. 3c). Consistently with dispersion theory, the rate of mass transport is quantitatively described by Fick’s 2nd law of diffusion ($R^2 = 0.99$), and is symmetric about the source[58]. In this dispersive regime, the meaning of the law’s parameters (especially the distance and the diffusion constant) can be different than in the case of molecular diffusion (Fig. 3d). If the distance is taken as the physical distance (~10 cm), then molecular diffusivity is replaced by a much larger “effective diffusivity” (e.g., $7.94 \times 10^{-7} \pm 0.531 \times 10^{-7}$ m²/s instead of $3.9 \times 10^{-10}$ m²/s in Figure 3c). If the diffusivity is instead fixed at the molecular diffusion value, the physical distance is replaced by a much smaller “effective distance” (e.g., 2.4 mm instead of 10 cm in Figure 3c), which scales almost linearly with the physical distance (Fig. 3e). The “signaling distance” represents the separation between two HOMEs that would show the same rate of mass transport by molecular diffusion alone.

While the analogies between dispersion and molecular diffusion can greatly facilitate the quantitative study of signaling in extended communities of organisms, the differences provide compelling opportunities. The signaling distance between HOMEs can be changed remotely: changing the input voltage to the pump from 0V to 8.7V reduces the signaling distance between HOMEs (Fig.3f) from 100 mm to 430 μm, (for comparison, the rhizosheath is ~1mm thick, the diffusive layer around the root is ~2mm, and the rhizosphere is ~10mm[59]). Different durations of the forward and backward pulse change independently the signaling distance between
Figure 3. Engineering dispersion for controllable mass transport between organisms. (A) Symmetric pumping scheme. (B) Displacement and voltage of pumping oscillations. (C) Timelapse of symmetric pumping and Fickian model fit to experimental data. (D) Schematic of molecular diffusion compared to dispersion. (E) Timelapse of distance pumping and Fickian model fit to experimental data. (F) Voltage as means to change “effective distance” lengths. (G) Asymmetries of pumping duration cause directionality in system. (H) Oscillatory flow snapshots in straight pipe with constriction extend mixing volume. (J) Contributions of features to dispersion.
one source and two sinks by effectively adding a controlled advective component: a
difference of 20 ms yields 50% differences in the signaling distances (Fig. 3g). Finally, the
oscillatory motion of the fluid reduces drastically the clogging and biofouling of the filters
between HOMEs when compared to unidirectional flows. Dispersion (7.5V, period=1s,
v=0.36 cm/s) of a bacteria solution (1.04±0.04 OD) through a 0.2 µm filter for 24 hours did
not clog the filter, while, by comparison, advective flow (v=4.1 cm/s, flow rate=1.3 cm³/s) of
a lower concentration (0.79±0.03 OD) of bacteria through a coarser filter (0.8 µm) lead to
clogging after only 15 secs (Fig. S21).

This mass transport is consistent with dispersion in oscillatory flows in the presence
of obstacles at large Womersley numbers (W~10)[60, 61]: the rate of mass transport is
independent of the diffusivity of the species being transported[61], is proportional to the
square of the displacement of the fluid column at each oscillation[62], and depends very
strongly on the frequency of the oscillations[63] (Figures S34 and S35). The effect of
dispersion is greatly amplified by the collective effect of obstacles in the oscillatory flow[64].
Figure 3H shows the effect of a single cylindrical bottleneck on a plug of dye: the bottleneck
causes jetting of the fluid, which leads to rapid mixing (over ~2 minutes) over a volume
(shown in blue) that is much larger than that displaced over each oscillation (shown in red).
Mass transport slows down dramatically after the complete mixing of the mixing volume,
with no observable mass transport after several minutes of oscillations.

The overlapping of mixing volumes is essential to the acceleration of mass transport
in HOME communities. The plot in Figure 3i shows the concentration of dye at the sink as a
function of time for two flow conditions: in one case the connection between source and
sink consisted of a straight tube (blue) so that the mixing volumes generated by the ports
between the HOMEs and the tubing would not overlap, and in the other it featured a
bottleneck in the middle (red), which creates a mixing volume that overlaps with the mixing
volumes at the end of the tube. In the first case, the dye spread from the source as far as
the mixed volume of the port allowed (up until halfway through the connection) and failed
to reach the sink. In the second case, the jet formed by the first obstacle is entrained in the flow field generated by the second constriction and the third (i.e., the sink’s port), resulting in rapid transport across the system. Using 3D numerical simulations of the fluid flow and concentration evolution (solved in a dimensionless form), we were able to confirm that oscillatory flow in a channel with just a single constriction can enhance effective diffusivity by two orders of magnitude (Supporting Information, Fig. S31).

The three modalities of mass transport (molecular diffusion, advection, and dispersion), and the four types of connectivities (empty, meshes, filters, and liquid membranes) allow for an exhaustive control on the means of signaling between HOMEs in a community (Table S1).

The ability to “plug and play” organisms in laboratory “ecosystems” by connecting HOMEs together and programming their signaling distance greatly facilitates the observation of phenotypic effects of plant biotic interactions. *Bacillus megaterium* interacts with plants through cytokinin signaling and promotes the growth of *A. thaliana* and *P. sativa*. [65, 66]. *B. rapa* plants were grown separately in two aerated 112 HOMEs. A 111 HOME colonized by *B. megaterium* was then connected to the *B. rapa* HOMEs in series by 0.45 μm filters. We reduced the signaling distances between the plants and the bacterium to 1.0±.02 mm and 2.0±.03 mm for one hour a day for three days using dispersion, generating a known gradient in exudate concentration (Fig. 4c). In terms of chemical signaling, this protocol placed the bacterium within the rhizosheath of one plant and at the edge of the diffusive layer of the other plant, leading to significant differences in biomass. When the bacterium was effectively in the rhizosheath of the plant, the biomass was significantly larger than for plants that were further away from the bacterium (41% for shoot, p=0.049, and 34% for root, p=0.014) and much larger than the control plants (63% for shoot, p=0.021, and 57 % for root, p=0.006). A significant advantage of such an
The experiment is that both plants were exposed to the same exact culture of bacteria, eliminating therefore the possible variability that would be associated with replicates that use different cultures. Plants placed closest to bacteria had a visibly more branched root system (Fig. 4d-e).

**Figure 4. Remote signaling between *B. rapa* and *B. megaterium.* (A) Schematic of mass transport. (B) Representative picture of plants 5d after stimulation with bacterial exudates. (C) Relative biomass of plants exposed to bacteria 10 cm and 20 cm away compared to plants not in contact with bacteria (error bar are 95% confidence intervals, n=4). (D) Representative root phenotype of plants 10 cm from bacteria. (E) Representative root phenotype of plants 20 cm from bacteria.
Conclusion

In conclusion, we have demonstrated that oscillatory laminar mixing and dispersion in millifluidic networks of modular habitats enables the independent control of the physical distance between organisms (cm-scale separation facilitates the independent control of their environments and the use of cm-scale organisms) and their signaling distance (mm-scale signaling distances are consistent with interactions in soil). This independent control of physical and signaling distances is a key step to enable the creation of model ecosystems for the quantitative study of collective behaviors in organismal communities. Furthermore, the approach is highly scalable, modular, simple, and frugal.

The advantages of this approach over currently available systems are significant. Co-cultures share their environment, so the rate at which they can exchange chemical signals is not accurately controlled and changes during the experiment due to the growth of the organism in space[44, 67]. This problem is sometimes addressed by exposing one organism to a solution of the exudate of the other[68, 69]. This approach cannot determine the mutual chemical response caused by the co-existence of the organisms, and is not applicable to communities of organisms (the number of control experiments would scale approximately with the factorial $n!$ of the number of organisms $n$). Approaches that use stirred habitats (e.g., bioreactors or hydroponic setups) separated by semipermeable meshes[70-73] establish a fixed distance between organisms, but also (i) introduce mechanical stresses on the organism[74-76], (ii) complicate the environmental control of the individual habitats due to their close contact[44], (iii) easily foul in the presence of microorganisms[77] leading to rapid changes in the effective rate of mass transport across the membranes, and (iv) cannot realistically establish large communities of organisms with independent and time-resolved control of their mutual connectivity and signaling rates.
Materials and Methods

Einstein Equation for estimation of diffusion time

\[ t = \frac{x^2}{2D} \]

\( t \) is the elapsed time since diffusion began, i.e. diffusion time, \( x \) is the mean distance traveled by the diffusing solute in one direction, and \( D \) is the diffusion coefficient of a solute in solution. Transport by diffusion will depend on the concentration gradient, the molecule size, and the medium the molecule is diffusing through.

Equation for calculation of Reynold's number

\[ Re = \frac{\rho * V * D_h}{\mu} \]

\( V \) is the fluid velocity, \( \rho \) the density of water at 25°C, hydraulic diameter of pipe (6.35mm), and \( \mu \) the dynamic viscosity of water at 25°C.

Description of flow velocity calculations

The flow velocity was measured using a system of two HOMEs connected with 10cm of straight polycarbonate tubing (1/4” ID, 3/8” OD). A 0.2 \( \mu \)m filter was mounted at each end of the polycarbonate tubing. Pumping was provided by a 6V peristaltic pump.

A 0.4 cm agar plug was created within polycarbonate tube. Agar was stained with blue food dye (Fisher Scientific) to provide a visual tracking of the plug in movies and facilitate image analysis.

The movement of the plug was recorded for 10s by a camera (Nikon 550D) at 33.36 fps from the top. Voltage was measured using a potentiometer (E-SUN®, #DT830). Each frame of the movies was exported as an image sequence. Displacements of plug were measured using ImageJ software. The results were plotted and fitted using Origin software with a sine (drift adjusted) function (Fig S25a-b):

\[ y = y_0 + A \sin \left( \pi \frac{x - x_C}{w} \right) + b * x \]
To calculate the velocity (Fig. S25c) the derivative is taken of the equation above

\[ \frac{dy}{dx} = A \cdot \frac{\pi}{w} \cos \left( \pi \frac{x - xc}{w} \right) + b \]

Fig. S25. Experimental characterization of the displacement and flow velocities induced by square function voltages applied to a peristaltic pump in a HOME system. 
A) Voltage trace obtained from the Arduino controller and input into the peristaltic pump. Lines are guides to the eye. 
B) Displacement measured from image analysis of movies of plug motion in a straight pipe. Lines are sinusoidal fits that account for linear drift. Drift is negligible outside of high voltages. 
C) Velocity plot vs time obtained by derivation of the fitted functions from panel b.

Description of dispersion fitting

Dispersion was characterized by estimating concentrations from colorimetric analysis of time lapses. A dye was introduced in the system. Color intensity was correlated with concentration, thereby allowing for the monitoring over time of mass transport without having to resort to chemical analysis. The calibration was performed with green food dye (McCormick) at known concentrations (6.5, 2.6, 1.3 mg/L). Each dye solution was introduced in a 111 HOME and was photographed in the same conditions of lighting and viewpoint that would be used during dispersion experiments. Using ImageJ software, the average pixel intensity (i.e., the darker the pixel, the higher the concentration of dye and the lower is the pixel intensity) within HOMEs was measured, plotted against the standard concentrations, and fitted to a linear function.

\[ y = -31.80283x + 253.4963 \]
The calibration curve (Fig S26) was then used to calculate concentration based on pixel intensity where the intercept represents when the concentration of dye is approximately zero.

![Calibration Curve](image)

**Fig. S26.** Representative calibration curve used to estimate dye concentrations in dispersion experiments.

**Fickian Equation Description**

Concentration vs. time plots were fitted using the analytical solution of Fick’s 2\(^{nd}\) law for two semi-infinite solids.

\[
q = -D \frac{\partial c}{\partial t}
\]

\[
\frac{\partial c}{\partial t} = \frac{\partial}{\partial x} \left[ -D \frac{\partial c}{\partial t} \right]
\]

Where the initial and boundary conditions are then:

\[
c = 0 \text{ at } t = 0 \text{ as } x \to \infty \quad s \quad x \to \infty
\]

\[
c = N \text{ at } x = 0 \text{ as } t \to \infty
\]
Solution with these conditions yields:

\[ c(x, t) = N \left[ 1 - \text{erf} \left( \frac{x}{\sqrt{4Dt}} \right) \right] \]

**Diffusion without dispersion**

Diffusion between HOMEs is effectively negligible due to their cm-scale distance. To test for this, we created a system with two connected HOMEs where one was the source of either dye or sodium chloride and the other was the sink. Known concentration of dye and NaCl were placed in the source (6·10^{18} molecules/m for the dye and 40 ppm for sodium chloride). The sink was sampled over the course of 10 days and the concentrations were measured by ICP to assess whether any diffusion had occurred. The data in Fig S27 shows that no significant mass transport of the solute had occurred. For up to 10 days.

![Fig S27. Molecular diffusion in HOME systems.](image)

**Fig S27. Molecular diffusion in HOME systems.** The plot shows the concentration of dye and NaCl in a sink HOME connected to a source HOME in the absence of any advection and dispersion over the course of ten days. No significant mass transport is observed.

**Simulation of dispersion in oscillatory flow**

We simulated the effect of oscillatory flow in a straight channel (Fig 3I bottom) as well as a constricted channel (Fig 3I top). We used an in-house finite element based simulation framework to model the Navier-Stokes equations for fluid flow, as well as the
advection-diffusion equation for the evolution of concentration. Both are solved in a dimensionless form.

We first solve the fluid velocity in the domain as a function of time (due to the imposed velocity conditions arising from the peristaltic pump) using the NS equation:

$$\frac{\partial u}{\partial t} + u \cdot \nabla u = -\nabla p + \frac{1}{Re} \nabla^2 u$$

The resolved velocity $u(x, y, z, t)$ (which is periodic in time) is then used to evolve the concentration using the advection diffusion equation

$$\frac{\partial c}{\partial t} + u \cdot \nabla c = \frac{1}{Pe} \nabla^2 u$$

Geometry and mesh: The geometry and actual dimensions of the pipe are used, non-dimensionalized and a 3D mesh created. The dimensionless diameter is 1, and the total length is 23.62. At the constriction the diameter is 0.5. The geometry and mesh both cases are shown in Fig 1. The mesh consists of linear tetrahedron with around 0.5 million elements for both (520160 for straight case, and 502235 for constriction case).

![Figure S28. Geometry and mesh for straight and constriction circular pipe](image)

Here, the Reynolds number $Re = 394$ in both cases, non-dimensionalized by reference velocity $u_0 = 0.0622 \text{ m/s}$, which is the maximum average velocity in the pipe in one period calculated from experiment. The Peclet number is taken to be $Pe = 10^6$.

Validation: We validate the periodic Navier Stokes solution framework by comparing with an analytical solution for a straight channel where a periodic pressure condition is applied. The analytical result is available in [chapter 4(6)], and we briefly restate it for clarity. Under a periodic pressure condition, $\frac{dp}{dx} = -\sin(t)$, the analytical solution of
horizontal velocity (for a 2D case) is

\[ u = -\left[ \left( 1 - \frac{f_1}{f_3} \right) \cos(t) - \frac{f_2}{f_3} \sin(t) \right] \]

Where

\[ f_1 = cc(k(y - h))cc(kh) + ss(k(y - h))ss(kh) \]
\[ f_2 = cc(k(y - h))ss(kh) - ss(k(y - h))cc(kh) \]
\[ f_3 = cc^2(kh) + ss^2(kh) \]

Where \( h \) is half of the distance between two parallel planes, and

\[ cc(x) = \cos(x) \cosh(x) \]
\[ ss(x) = \sin(x) \sinh(x) \]

\[ k = \sqrt{\frac{1}{2} Re} \]

In Figure 2, we compare simulation results with the analytical result at different time points within one period of pressure oscillation for \( Re = 20 \). The results confirm that the numerical model is faithfully replicating the desired oscillatory physics.

**Figure S29.** Comparison of numerical and analytical horizontal velocity as a function of height at dimensionless \( t = \frac{\pi}{2}, \pi, \frac{3\pi}{2}, 2\pi \)
Navier Stokes for periodic flow conditions: We used the validated model to simulate periodic flow fields for the two geometries shown in Fig 1. We apply velocity boundary condition, \( u = C \sin(\omega t) \), with \( C \) and \( \omega \) calculated from experiment to both inlet and outlet. The velocity (initially set to be quiescent fluid) shows a periodic profile. We probe the horizontal velocity at four points, 0.25\( L \), 0.5\( L \), 0.75\( L \), and 0.95\( L \) along the pipe axis to track this periodic behavior. This is shown in Figure 3.

![Horizontal velocity probe](image)

**Figure S30.** Horizontal velocity probe at 0.25\( L \), 0.5\( L \), 0.75\( L \) and 0.95\( L \) for (a) straight case. (b) constriction case

We next pick one full period of the velocity field, and use this periodic field to solve the convection-diffusion equation. We assume a source of the dye at the left boundary and hence set the boundary condition as \( c(x = 0, t) = 1 \) at the left inlet; and assume a sink at the right end and set no flux the right boundary. All walls have the standard no-flux boundary (i.e. no penetration). We probe the cross-section averaged concentration at 0.95\( L \) (towards the right end of the domain) and plot this average concentration as a function of dimensionless time in Figure 4. We clearly see the effect of the constriction on enhancing the effective diffusion. We next fit these curves to calculate the effective diffusivity.
The inclusion of the constriction enhances the effective diffusivity of the system by two orders of magnitude from a $D \sim 10^{-1}$ to $D \sim 10^1$. This is much higher than the (non-dimensional) molecular diffusivity (i.e. peclet number) of $\frac{1}{Pe} = D \sim 10^{-6}$.

We also show videos of the concentration evolution for these two cases (movie S3 and S4). We can clearly see that the constriction in the middle helps to propagate the concentration faster due to the formation of vortices and a jetting behavior (Figure 5).

**Dependence of effective diffusivity on diffusion coefficient**

Bovine hemoglobin (0.010M, Sigma, $D_{\text{hemoglobin}} = 3.4 \times 10^{-12}$ (7)) and Fast Green dye (0.009M, Fisher Scientific, $D_{\text{fastgreen}} = 3.9 \times 10^{-10}$) were used to analyze the influence of molecular diffusivity coefficients on mass transport by dispersion. The testing system was designed to ensure equal pumping force for the dispersion using both types of molecules (Figure S33). The calculated effective diffusivity of hemoglobin,
$D_{\text{hemoglobin, effective}} = 4.30 \times 10^{-7}$, matches the effective diffusivity of Fast Green dye, $D_{\text{fastgreen, effective}} = 4.35 \times 10^{-7}$ (Figure S33). This suggests that, within the range of molecular diffusivities we tested and the oscillatory flow parameters we used, dispersion does not depend on molecular diffusivity.

**Figure S33.** (A) Experimental design for molecules effective diffusivity comparison. (B) Concentration vs time curves of Fast Green dye and Hemoglobin under dispersion.

**Dependence of Effective Diffusivity on Displacement and Oscillation Frequency**

**Figure S34.** Dependence of effective diffusivity on the square of the displacement (a) and on the frequency (b) of the oscillation of the fluid column.
Control of Signaling Moieties by Combinations of Mass transport modalities and semi-permeable membranes

The combination of the three types of mass transport (diffusion, advection, and dispersion), along with the choice of permeable/semi-permeable membranes (open, mesh, filter, liquid) allows for a nearly complete selection of what species/organisms are allowed to transport, move, or grow in between HOMEs. The table below provides a guide on selecting the appropriate type of transport and connectivity depending on the hypothesis that is being tested about signaling.

<table>
<thead>
<tr>
<th>Connection type</th>
<th>Mass transport mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>diffusion</td>
</tr>
<tr>
<td>open</td>
<td>microbes, roots</td>
</tr>
<tr>
<td>mesh</td>
<td>microbes</td>
</tr>
<tr>
<td>filter</td>
<td>nothing</td>
</tr>
<tr>
<td>liquid</td>
<td>roots</td>
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Table S1. Control of signaling moieties

References


CHAPTER 4. FROM PETRI DISHES TO MODEL ECOSYSTEMS

Modified from a paper submitted

Oskar Siemianowski†, Kara R. Lind†, Xinchun Tian†, Matt Cain†, Songzhe Xu‡, Baskar Ganapathysubramanian‡, Ludovico Cademartiri†, ‡, ‡

1 Department of Materials Science & Engineering, Iowa State University of Science and Technology, 2220 Hoover Hall, Ames, IA, 50011
2 Department of Chemical & Biological Engineering, Iowa State University of Science and Technology, Sweeney Hall, Ames, IA, 50011
3 Ames Laboratory, U.S. Department of Energy, Ames, IA, 50011
4 Department of Mechanical Engineering, Iowa State University of Science and Technology, Ames, Iowa, 50011
† These authors contributed equally to this work.

Abstract

Model ecosystems could provide significant insight on the evolution and behavior of real ecosystems. We discuss the advantages and limitations of common approaches like mesocosms. In this context, we highlight recent breakthroughs that allow for the creation of networks of organisms with independently controlled environments and rates of chemical exchange.

Introduction

We want to start with the following “food for thought”. Arguably, one of the greatest advances in chemistry was the development of glassware. It enabled the maturation of chemistry into a quantitative science by allowing to control the conditions of reactions (e.g., temperature and atmosphere composition) with modular, transparent setups that could be easily standardized and replicated. Similar statements could be made about the role of Petri dishes in the development of microbiology.

Here we convey this thought to plant science which could greatly benefit from the development of a “glassware” for biology: controlled, modular, scalable, transparent, connectable habitats for growing plants and microbes. We argue that such tools could enable the understanding of interactions in communities of organisms by providing a repeatable and quantitative experimental platform. A predictive
understanding of these interactions and their collective effect on plants is essential for (i) breeding for specific environments/ecosystems (e.g., perennial cover crops[3]), (ii) understanding the genetic modulation of these interactions (e.g., quantitative trait loci mining[4]), and (iii) understanding how the environment controls the response to disease and stress (e.g., sudden death syndrome in soybean[5]).

Ecosystems – like the human organism – are complex systems whose behavior is often emergent, i.e., they are more than the sum of their parts. Therefore, holistic and reductionist approaches serve complementary roles in the study of complex systems. Holistic approaches (e.g., field work) capture behaviors and responses of real systems, but lack well defined control experiments, repeatability, and the knowledge of all variables. By contrast, reductionist approaches, do not usually seek to predict the behavior of the real system, but can provide insight on (i) cause-effect relationships (i.e., how the system responds to stimuli[6]), (ii) critical phenomena (i.e., how and when the state of the system can suddenly change[7]), (iii) feedback loops (i.e., what processes are interdependent[8]), and (iv) scaling behaviors (i.e., how the system’s behavior changes with its size and complexity[9]).

Results and Discussion

Creating Model Ecosystems

Model systems like tissue cultures and model organisms have advanced plant science greatly, but comparably little work has been done to create “model ecosystems” to understand plant communities and the rhizosphere. What is a model ecosystem? The development of an ecosystem depends on the environment and the chemical (e.g., signaling) and physical (e.g., shading) interactions between its members. Therefore, a model ecosystem should provide independent control of the environmental conditions of each organism (e.g., nutrients and water availability, humidity, light, and temperature) and of their mutual interactions (e.g., rates of flux exchange, distance of signaling).

Achieving independent control of these factors is technically difficult for two main reasons. (i) The environment of an organism is affected by its interactions (e.g.,}
competition for nutrients). This raises the question, how to allow interactions between the organisms, if we are to control their environment? (ii) The characteristic signaling distances between organisms are often limited to the millimeter scale by the slow rate of diffusion in liquids (e.g., the rhizosphere is ~2-5 mm thick). Because the signaling distance is smaller than the size of the average root system, different parts of the same root system are at significantly different distances from their neighbors and therefore will experience different concentrations of exudates from them. This raises further questions: How to physically separate cm-scale organisms, such as plants (so that we can control their environments), if we have to keep them nearly in contact? And, importantly, how can we achieve the above for dozens of organisms independently, with a simple, robust, and frugal approach that can be widely adopted by the scientific community?

**Available Tools**

Current legacy tools (e.g., Petri dishes, Magenta boxes, rhizotrons) are exceptionally simple, standardized, inexpensive, and benefit from decades of protocol development, but were not designed for the creation of model ecosystems. They can easily produce micro- and mesocosms (Figure 1A-B) – simplified, smaller versions of real ecosystems – that do not control the interactions between organisms, nor their individual environments[10]. Engineered environments such as microfluidic circuits[11] physically confine the organisms and control mass transport at the micron scale, but are difficult to scale to the size of plants (especially crops). Physical networks of independently controlled habitats are another approach to the creation of model ecosystems (Figure 1A-B). Every habitat physically confines an individual organism but allows it to chemically interact with the neighboring habitats.

In principle, the network approach has significant advantages over the microcosm approach: (i) the size and environment of each habitat can be controlled independently, (ii) the distance between the habitats (Figure 1C), and therefore the relative rate of chemical signaling (Figure 1D), can be determined (if the organisms are physically
confined by semi-permeable membranes), (iii) organisms can be plugged in and out of the ecosystem to provide internal control experiments, (iv) each organism can be independently phenotyped, (v) large communities can be analyzed by network theory approaches to discover, quantify, and understand collective behaviors).

Figure 1. Different approaches to the creation of model ecosystems: microcosms vs physical networks. 
(A) Differences in the structure of the model ecosystems: microcosms share the same environment, while physical networks confine the organisms in separate but chemical connected environments. (B) Representative examples of microcosm[1] and physical network model systems[2]. (C) Qualitative plots describing the evolution over time of the distances between organisms in the two approaches: microcosms show a wide range of distances between different parts of the same organisms (see red arrows in panel A), while network distances are constant and determined a priori. (D) Qualitative plots of the concentrations of exudates at organism 1 as a function of time as a result of the distances between the organisms: microcosms show a wide range of interaction kinetics between different parts of the multiple organisms due to the wide range of distances, while networks should provide predictable diffusion kinetics due to the precisely defined distances.
An apparently fundamental issue with the network approach is that the distances between habitats of cm-scale organisms (1-5 cm) are significantly larger than the diffusion-limited signaling distance (1-5 mm): the organisms confined to nodes of cm-scale networks would not be able to exchange signaling molecules by diffusion at a rate that is comparable to the one observed in their ecosystems, therefore rendering the model ecosystem useless.

A simple solution to this mass transport bottleneck was recently reported[2]. Oscillatory flows in these networks of habitats lead to a diffusion-like transport of chemicals whose rate can be controlled over several orders of magnitude (Figure 2A). Controlling the rate of diffusion between habitats is mathematically analogous to controlling their distance. In the conditions we reported, this effective distance could be made smaller than 1 mm. In other words, organisms could be made to signal to each other as if they would be only 1 mm apart (i.e., as if they would be in each other’s rhizosphere) even though they were confined to independently controlled environments. This capability allowed us to quantify the effect of distance (1 mm, 2 mm and 10 cm) on the phenotypic response of Brassica rapa to Bacillus megaterium (cf. Figure 2B-C). Because this mass transport can be predicted by the laws of diffusion, the relative amounts of exudates exchanged at the different distances could be quantified and correlated with biomass.

An important side effect of these oscillatory flows is that they minimize biofouling of the semi-permeable membranes that we used to confine the colonies. In the presence of unidirectional flow, the filters clog in seconds, while our oscillatory flows persist for hours without clogging.

**Conclusion**

At least for a time, model ecosystems in the form of physical networks of habitats are bound to be more complex, expensive, time consuming than some of the co-culture protocols commonly employed in laboratories (e.g., partitioned Petri dishes).
They are likely to require simple programming of controllers, some understanding of mass transport, and entirely new standardized protocols.

Figure 2. Control of signaling distance in a model ecosystem. (A) Time lapse of diffusion-like mass transport of a green dye in a series of three habitats connected in series and undergoing oscillatory flow (left). The rate of mass transport can be fit with the diffusion equation (right), using an effective ("signaling") distance between habitats that is much smaller than the physical distance (2.4 mm instead of 10 cm). (B) Setup to quantify rhizosphere interactions as a function of distance between *B. megaterium* and *B. rapa* plants using a physical network approach and oscillatory flows to control the signaling distance. (C) Quantitative effect of the distance between colony and plant (1 mm and 2 mm) on the plant biomass, and its correlation with the relative amounts of exudates, calculated from the diffusion equation.
By contrast, we believe the fundamentally new possibilities offered by this network approach to model ecosystems will provide new opportunities for the communities of plant science, microbiology, and ecology. We suggest here five general examples for discussion. (i) **Quantifying interactions and microbiome evolution within the rhizosphere:** Habitats containing real soil microbiomes can be placed at different signaling distances from one or more plants. The evolution of the individual microbiomes can be then correlated with the distance from the root system, while observing the effect of the microbiome on the root phenotype. (ii) **Culturing unculturable bacteria:** Specific colonies can be exposed to exudates from established microbiomes in real soil while being physically confined from them, similarly to what was shown in work of Epstein and colleagues[12]. (iii) **Collective responses to stimuli.** A community of organisms can be stressed/stimulated in space (all organisms or a subset of the organisms) and/or in time (e.g., organisms can be plugged in or taken out, environmental variables can be changed for a short time, toxins can be flowed through the system for a determined time) and the local and global response can be phenotyped over time. Well-defined control experiments can quantify the role of the network’s connectivity on the stress response. (iv) **Volatiles vs non-volatiles signaling.** The independent control of the above ground and below ground environment enables the distinction of signaling mechanisms. (v) **Interactions with pests.** Interactions with pests can be easily introduced in these communities thanks to the cm-scale dimensions.

**References**

CHAPTER 5. A SIMPLE AND VERSATILE 2-DIMENSIONAL PLATFORM TO
STUDY PLANT GERMINATION AND GROWTH UNDER CONTROLLED
HUMIDITY

Modified from a paper submitted

Tom Sizmur†§, Kara R. Lind‡, Saida Benomar‡§, Hannah VanEvery†, Ludovico Cademartiri†‡,*

† Department of Materials Science & Engineering, Iowa State University, Ames, IA
‡ Ames Laboratory, US Department of Energy, Iowa State University, Ames, IA
§ Department of Chemical & Biological Engineering, Iowa State University, Ames, IA
§ Department of Sustainable Soils and Grassland Systems, Rothamsted Research,
   Harpenden, Hertfordshire, AL5 2JQ, United Kingdom

Abstract

We describe a simple, inexpensive, but remarkably versatile and controlled
growth environment for the observation of plant germination and seedling root growth
on a flat, horizontal surface over periods of weeks. The setup provides to each plant a
controlled humidity (between 56% and 91% RH), and contact with both nutrients and
atmosphere. The flat and horizontal geometry of the surface supporting the roots
eliminates the gravitropic bias on their development and facilitates the imaging of the
entire root system. Experiments can be setup under sterile conditions and then
transferred to a non-sterile environment. The system can be assembled in 1-2 minutes,
costs approximately 8.78$ per plant, is almost entirely reusable (0.43$ per experiment
in disposables), and is easily scalable to a variety of plants. We demonstrate the
performance of the system by germinating, growing, and imaging Wheat (Triticum
aestivum), Corn (Zea mays), and Wisconsin Fast Plants (Brassica rapa). Germination
rates were close to those expected for optimal conditions.
Introduction

Approximately 97% of the calories consumed by humans originate from plants [1]. Recent estimates indicate that the food supply will have to increase by approximately 70% by 2050 to match demand [1]. However, even optimistic estimates predict only a 50% increase in crop yield by 2050 [2].

Improving our understanding of seed germination and root growth could be necessary to ensure our food security in the future, since the germination, emergence, and early establishment of seedlings have a large effect on agricultural yields, especially if below a critical level [3]. Low germination rates reduce crop density, which results in indirect yield loss. Late emergence can result in poor plant performance and a direct yield loss [4], because roots are inadequately established and have less access to water and nutrients during later stages of vegetative and reproductive growth.

Tests of seed viability and vigor typically employ paper to act as a support and to supply moisture: seeds are placed over moist germination paper (and often covered with a second sheet) and incubated. A germination table (also known as a Copenhagen table or Jakobson apparatus) can germinate several seeds simultaneously under one set of conditions [5], [6]: filter paper wicks moisture from a temperature-controlled water tank and provides a flat, horizontal surface on which germination can be observed. However, germination tables are expensive, not universally available, and do not provide control of conditions to individual replicates. Furthermore, they are not ideally compatible with – and never used for – the study of plant root growth. Most plants grown for research purposes are transplanted at least once after germination.

Roots are responsible for the vast majority of the water and nutrient supply to the plant [7], they establish synergic interactions with soil biota [8], [9], and they anchor the plant to the soil [10]. By these functions, the roots influence the growth of the plant and its resilience against environmental stresses such as drought. Root architecture (i.e. its size and structure) plays a fundamental role in plant productivity and crop yield [11].
Nonetheless, roots and their development are one of the most complex and relatively unexplored aspects of the food supply problem [12].

Seedlings are grown in granular media (e.g., soil, sand, perlite, vermiculite) or homogeneous media, such as water (hydroponics), air (aeroponics), or gels (e.g., agar, gelatine, gellan gum). Gels provide a 3D growth environment for the roots, but they otherwise poorly represent the mechanical and structural properties of soils [13], and may expose plants to anoxic conditions [14]. Analysis of the size and structure of a 3D root system requires relatively sophisticated equipment and cumbersome image analysis [15]. Granular media (e.g., soil, sand, vermiculite) is structurally closer to soil, but is opaque to most forms of radiation. The imaging of root systems in those environments requires expensive equipment (X-ray computed tomography or magnetic resonance imaging [16], [17]) that is not widely available, currently has low throughput (individual scans can take hours), and cannot routinely or accurately distinguish live roots from dead organic matter [18]. Roots can be imaged growing against a transparent surface in soil-filled 2D root-boxes called rhizotrons [19]. However, even when tilted at a 43° angle to encourage the roots to grow against the transparent surface, less than half of the total root length is visible [20], root density is overestimated [21], root development is affected by gravitropism, and the soil/glass interface is unlikely to be representative of real soil structure.

The study of both germination and root development in the same environment is experimentally and logistically difficult, because of the lack of convenient and yet highly controlled and capable environments in which to study these processes [22]. Conditions for experimental plant germination and growth must be precise and uniform, because plants are highly sensitive to environmental conditions, and exhibit phenotypic plasticity to a vast array of abiotic stimuli [23]. Some of these conditions (e.g. temperature, light, humidity and CO₂ concentration) can be controlled using growth chambers [24]. Growth chambers are not ideal environments to study the effect of humidity on plant development because (i) they cannot control humidity of individual
replicates, (ii) they expose the plant to the atmosphere and potential contamination, and (iii) they are expensive and not universally available. Therefore, laboratory studies of plant germination and growth under controlled humidity conditions typically require a large upfront investment. These barriers are bound to inhibit or prohibit investigators from other disciplines or developing nations from entering into this area of science.

We describe in this paper an experimental setup for the study of germination and root development of a variety of plants (as shown here, *Brassica rapa*; Wisconsin Fast Plants; Astroplants, *dwf1* [25], *Triticum aestivum*; Wheat, and *Zea mays*; Corn). The platform displays the following capabilities and characteristics: (i) It constantly exposes the plant to a nutrient solution and to a controlled humidity (ranging between \(\sim 56\%\) and \(\sim 91\%\) in each setup). (ii) It can be used on any laboratory bench, as long as uniform illumination and temperature are provided. (iii) It is composed of reusable or inexpensive parts. (iv) It is scalable to virtually any plant size. (v) It allows imaging of the shoot and root. (vi) It eliminates the gravitational bias on root development by growing the roots on an horizontal and flat 2D surface, which facilitates the imaging and analysis of the entire root system architecture.

**System Design**

The assembly of the platform is shown in Figure 1a. It consists of 8 steps that can be completed in approximately 1 to 2 minutes (see Supporting Information S1 for a detailed description and Movie S1 and Movie S2 for a video demonstration) and result in the self-contained plant growth environment shown in Figure 1b. The design of the platform was constrained by a stringent set of conditions. **Delivery of nutrients and moisture to the seed/plant.** In our setup, the seed (*B. rapa, T. aestivum, or Z. mays*) is supported on a flat sheet (the “growth sheet”) of filter paper (Whatman #1). The growth sheet lies on top of a larger sheet (the “pump sheet”) of filter paper (Whatman #1) that wicks nutrient solution from an underlying reservoir. The pump sheet imbibes the growth sheet with the nutrient solution. Coating the newly sown seed with a hydrogel droplet (50 µl of gellan gum) improves germination rates: the hydrogel draws water
from the filter paper and ensures the seed is moist without eliminating the access to oxygen. **Compatibility with both germination and growth.** The setup is easily scalable. Figure 1b-d show that three plants with different seed size can be germinated in our platform. The overall scale of the experiment can be controlled to match the size of the plant after the intended growth period (see Supporting Information S1).

**Figure 1. Schematics and picture of plant germination platform.** Scheme of the assembly of the plant germination and growth platform. Pictures of the assembled setup growing (b) *Brassica rapa*; Wisconsin Fast Plants; Astroplants, dwf1, (c) *Triticum aestivum* (Wheat), and (d) *Zea mays* (Corn). (e) Picture of a Corn seedling held at 90 degrees on paper, demonstrating the anchoring of the roots to the filter paper support.

Plant roots anchor to the filter paper. As shown in Figure 1e, plants grown for 2-3 weeks can be turned sideways without toppling over. **Control of humidity.** Supersaturated salt
solutions in a closed environment establish an atmosphere of known relative humidity [26]. Different salts controlled the relative humidity of the air 5 cm above the growth sheet between \( \sim 56\% \) and \( \sim 91\% \) at 25°C (Figure 2a). The external container (containing the salt solution) is never in contact with the nutrient solution, so neither the salt nor the container can contaminate the paper on which the plant is grown. **Exclude the influence of gravity on the direction of plant root growth.** Gravity affects root growth by creating a gradient of auxin across the root tip. Auxin is a hormone that inhibits the expansion of root cells. A gradient of auxin across the root cross-section cause the root to bend due to differential expansion of the tissue [27], [28]. The gradient of auxin is determined by the angle between the root tip and the gravitational field: if the root is pointing downward the angle is zero, there is no cross-sectional gradient of auxin, and the root grows mostly straight. Therefore, gravitropism cannot influence the direction of root growth in a horizontal plane. Gravitropism typically dominates the early stages of root growth and can complicate the assessment of the influence of other stimuli (e.g. water or nutrient gradients) on the development of roots: as the root grows, the angle it makes with the gravitational field can change, therefore changing the distribution of auxin in time and space. Roots grown on a horizontal surface still develop a gradient of auxin (the gravitational field is still present), but it remains constant and homogeneous across the whole root system. Therefore, a flat, horizontal surface provides a convenient way to monitor root development in response to stimuli other than gravity, since the effect of gravity is not removed but is constant. Our setup provides a flat horizontal surface by overlaying the paper on a glass slide – which provides a flat surface – supported on a platform constructed from LEGO bricks – which ensures the surface is horizontal. The remarkable precision of LEGO bricks (molds have a tolerance of 5 µm or less [29]), together with their convenience, reusability, modularity, transparency, low cost, chemical inertness, and compatibility with autoclaving makes them nearly ideal building blocks for the rapid prototyping of structurally precise biological environments in the mm to cm scale [30]. Several setups can be arranged on a single flat leveled surface to ensure that all growth
sheets in all setups are horizontal. Other options to control for gravitropism exist (e.g., using agravitropic mutants [31], growing plants in space [32] or in a clinostat [33]) but are considerably more demanding. **Low cost.** There is a growing requirement to consider the cost of science from the beginning [34]. Our setups cost $8.78 per plant, of which only $0.43 is for disposable items. The setup does not require any equipment unless sterilization is required (in which case a class II biosafety cabinet is sufficient). **High throughput.** High throughput plant experiments are typically conducted on gel in Petri dishes that (i) are capable of processing thousands of individual seeds/plants per week or month [35], [36], [37], (ii) can be setup in less than 1 minute, and (iii) can be stacked so that up to 5000 per m² [35] can be fit in growth chambers. Our experimental units can be assembled in ~1 to 2 minutes (see Movie S2 for a demonstration) and each of the parts can be prepared (i.e., autoclaved, cut, dissolved) in batches (we can assemble from scratch approximately 100 setups per person, per day). Each setup has a footprint of 60 cm², or ~167 units per m². Although our setup does not have the throughput of Petri dishes, it provides control over humidity and is compatible with much larger plant sizes. **Sterile.** Plant science research requires the growth and development of plants under sterile conditions [38]. All components are easily sterilized (the LEGO bricks, plastic cup, glass slide, MAGENTA box, gellan gum, salt and nutrient solutions are autoclaved, while the paper and plant seeds are soaked with 70% ethanol). After our setup is assembled in a sterile environment and sealed within the MAGENTA box, it can be transferred to a non-sterile environment without contamination. **Suitable for any laboratory bench.** The advancement of life science in the 21st century will require contributions from other disciplines [39] and developing world laboratories [40]. Facilitating these collaborations will require methods compatible with any laboratory bench in the world, regardless of discipline or resources. Our experiments were performed in handmade chambers (see Supporting Information S1) constructed from a wooden frame and aluminum foil. The purpose of the chambers was to provide uniform illumination of the plants, and prevent the establishment of thermal gradients. Plants were grown in our growth chambers underneath an array of
225 white LEDs so that the plants would receive \( \sim 9000 \) lumens. **Capable of supporting increasing levels of complexity.** The support of the seed is filter paper. This choice was influenced by the recent reports of ‘lab-on-paper’ technologies that have been developed to provide fluid manipulation [41], chemical reactions [42], and environments for microorganisms and cell cultures [43] in paper substrates. The combination of the platform presented here with the tools of paper microfluidics is beyond the scope of this communication, and will be the focus of future publications.

**Figure 2. Performance of the germination and growth platform.**

- **a)** Relative humidity in the setup as a function of the salt used to form the supersaturated solution in the reservoir. Error bars are 3 standard errors, \( n = 3 \).
- **b)** Plot of the maximum germination rates obtained for Fast Plants, Wheat and Corn in our platform, compared to optimal germination rates reported by our seed source.
Results and Discussion

The performance of the germination and growth environment was assessed by (i) its control over relative humidity, and (ii) its ability to yield high germination rates. Figure 2a shows the relative humidity (RH) measured 5 cm above the surface of the growth sheet (the approximate height of the cotyledons of a *B. rapa* plant after the hypocotyl straightens), as a function of the super saturated salt water solution held in the external container. All measurements were performed at 20°C: the measurements were made on a laboratory bench where the temperature was not stabilized (we estimate the error on the temperature to be ~2°C). The RH can be controlled between 90.6 ± 0.9% (with KSO₄, error is three standard errors, n=3) and 56±8% (with LiCl, error is three standard errors, n=3). The difference between these measured RH values and those expected from the respective saturated solutions – a super saturated LiCl solution in water should establish a RH of 12% – probably results from the fact that the atmosphere within the enclosed setup is exposed to both the saturated salt solution and the nutrient solution. Thereby, while the saturated salt solution is absorbing water from the atmosphere, reducing the RH, the nutrient solution is evaporating, increasing the RH. The steady state results in the observed RH. Of course, the above explanation implies that the observed RH will not only depend on the salt solution chosen to reduce RH, but also on the ratios between the areas of the exposed surfaces of the saturated salt solution and the nutrient solution in the setup. Broader ranges of RH should be accessible by changing the ratios of the exposed surfaces. The evaporation of the nutrient solution and the absorption of water by the saturated salt solution should increase the concentration of the nutrient solution over time. Our measurements indicate that the change is not detectable over the course of 15 days, at least when using NaCl as the saturated salt solution (see Supporting Information S1).

Figure 2b shows the germination rates for *B. rapa*, *T. aestivum*, and *Z. mays*, in our platform, compared to the germination rates reported by our seed source. The rates we obtained are remarkably close to the expected ones, especially considering that
minimal effort was put into optimizing standard seed handling protocols for our platform (see Supporting Information S1 for details).

The ability to visualize whole root systems will be increasingly important for understanding the responses of roots to stimuli, and breeding plants with desirable traits. Figure 3 demonstrates the use of our setup for the quantitative analysis of the whole root system of a *T. aestivum* seedling. The root system was photographed from above after the shoot is removed (Figure 3a). We increased the contrast of the image (details in Supporting Information S1) and removed the seed from consideration by superposing a white colored circle over it (Figure 3b). The resulting image was then analyzed with standard root-analysis software (in our case WinRhizo) yielding phenotypic data for the whole root system (Figure 3c).

![Figure 3](image)

**Figure 3.** Example of root image analysis performed on roots grown on our experimental setup. a) Top-view photograph of a Wheat (*Triticum aestivum*) seedling grown for 7 days after the shoot has been removed. b) Modified version of the photograph in panel a) after the seed has been digitally removed, the color has been made black and white, the contrast has been maximised. c) Table of selected root parameters obtained by the analysis of the image in panel b) by WinRhizo.
Conclusion

We addressed in this communication the challenge of providing a simple, inexpensive, and yet reproducible and capable apparatus for the observation of germination and seedling growth in sterile environments with controlled humidity.

The system we designed combines tools that are commonly used by plant scientists (e.g., filter paper, MAGENTA boxes) and others that are not (e.g., LEGO bricks) to fulfill a number of strict design requirements which include low cost, simplicity, structural precision, control over humidity, scalability to any plant size, and high throughput. Specifically, we demonstrated that the setup, as it is designed, (i) can grow plants for weeks, despite its planar geometry (the plants do not topple over but balance and anchor themselves with their roots), (ii) provides a constant supply of water, to the seed and root system, (iii) maintains a constant relative humidity between 91% and 56%, (iv) is capable of germination rates comparable to those expected from the species we tested (B. rapa, T. aestivum, and Z. mays), (v) enables sterile plant growth experiments in a non-sterile environment, (vi) facilitates imaging and image analysis of whole root systems, and (vii) cost 8.78$ (of which only 0.43$ are in non-reusable items) to buy and 1-2 minutes to assemble.

This platform represents one element of a series of integrated, simple, and reproducible tools that our group will be introducing to create highly controlled mm and cm-scale biological environments for plants and other organisms.

Materials and Methods

Relative humidity of different salts

Prepare in a beaker (glassware) a super-saturated solution of salts by dissolving the amount of salt listed below in 100 ml of Milli-Q water.

Potassium sulfate (50 g)

potassium nitrate (164.4 g)

potassium chloride (80 g)
sodium chloride (72 g)
potassium carbonate (224 g)
calcium chloride (300 g)
magnesium chloride (220 g)
lithium chloride (115 g).
Pour the powder little by little with stirring at room temperature. Use the same protocol for plant germination. Cover the bottom of MAGENTA box by the one of the salts to test. Add 50 ml of this salt. Use a MAGENTA box lid with hole.
Introduce the hygrometer to measure the humidity (see figure S15 control). Report the value indicated by the hygrometer when the temperature is stable.
Then, in the same box, put the cup (reservoir) with 25 ml nutrient solution and follow the same steps to put the growth paper. Introduce the electrode to measure the humidity (see figure S15 experiment). Report the value indicated by the relative humidity meter when the temperature is stable.

![Figure S15](image)

**Figure S15.** Measure of relative humidity (%), control without pump and growth paper and the experiment with pump and growth paper.

**Concentration of the nutrient solution**

The concentration of nitrates and ammonium ions were characterized in the nutrient solution after 0, 1, 6, 7, 8, 10 and 15 days, when using NaCl as the supersaturated salt. The results (n=3) are reported in Figure 15.
Figure S16. The concentration of nitrates and ammonium ions measured in the pump sheet does not significantly change over time

Image Analysis

The image of the corn seedling on the paper sheet (Figure 3a) was first converted to black and white, then processed by the “HDR-ish” filter of Google Picasa 3.0. The resulting image was subjected to a maximization of the Highlights and Shadows knobs, still in Picasa, resulting in a nearly binary image. The roots were then cut with a “Magic Wand” tool in Adobe Fireworks CS3 and pasted in a separate document with white background. The seed was covered and removed from further consideration by superposing a white circle over it. The resulting image was processed by WinRhizo yielding the root parameters described in Figure 3c.

Limitation and Future Work

The platform described here is remarkably flexible, versatile, scalable, and, as future work from our group will demonstrate, capable of remarkable control over the physico-chemical parameters of the environment surrounding a plant and its root system. Nonetheless, it does present certain limitations, some of which are fundamental and some of which not so. Temperature control. The setup, in its current design stage, does not allow for the independent control of temperature. Each experiment can,
however be freely moved from one controlled temperature environment to another without (i) physically disturbing the plant, (ii) affecting the atmospheric composition, (iii) compromising sterility, or (iv) affecting the orientation of the root with respect to gravity. *Germination at an angle*. While the root does anchor to the filter paper, the germination of species with large seeds cannot at present be accomplished at large angles (e.g., 90°) with the current design: the seed is not sufficiently adhering to the filter paper. This limitation could be easily overcome by overlying the seed with a second strip of paper, using the adhesion between this strip and the growth paper to hold the seed on the paper. *Inherently 2D geometry*. While the 2D geometry of the setup is remarkably convenient for the study of root development (e.g., localized application of stimuli, imaging and prototyping are substantially easier in a 2D configuration), 3D environments need to be developed as well to test any hypothesis originating from 2D experiments. Other work from our group [30] and others [45] is attempting to create 3D soil mimics for this purpose. *Incapable of generating very low humidity*. The current limit of the setup is ~55% RH, which is still high for any experiment that attempts to recreate very low humidity conditions. We believe this restriction is not a fundamental limit of the technique, and that RH values of ~10% are achievable with this same approach. *Instability of the nutrient concentration over time*. The current setup is vulnerable to relatively large changes in the concentration of the nutrients at the growth sheet due to the slow evaporation of the nutrient solution from it. Given the limited transport guaranteed by the pump sheet, the small volume of solution held in the pump sheet, and the relatively large distance between the top of the pump sheet and the nutrient reservoir, the concentration of nutrients can change over time. Modifications are being experimented with to provide not only a minimization of salt accumulation in the growth sheet but an effective constancy in the concentration of nutrients in contact with the root system. These modifications will require extensive characterization that is beyond the scope of this manuscript.


CHAPTER 6. PLANT GROWTH ENVIRONMENTS WITH PROGRAMMABLE RELATIVE HUMIDITY AND HOMOGENEOUS NUTRIENT AVAILABILITY

Modified from original paper

Kara R. Lind1, Nigel Lee2, Tom Sizmur1,3,5, Oskar Siemianowski1, Shawn Van Bruggen4, Baskar Ganapathysubramaniam2, Ludovico Cademartiri1,4,5*

1 Department of Materials Science & Engineering, Iowa State University of Science and Technology, 2220 Hoover Hall, Ames, IA, 50011
2 Department of Mechanical Engineering, Iowa State University of Science and Technology, Black Engineering, Ames, IA 50011
3 Department of Geography and Environmental Science, The University of Reading, Reading, RG6 6DW, UK
4 Department of Chemical & Biological Engineering, Iowa State University of Science and Technology, Sweeney Hall, Ames, IA, 50011
5 Ames Laboratory, U.S. Department of Energy, Ames, IA, 50011

Abstract

We describe the design, characterization, and use of “programmable”, sterile growth environments for individual (or small sets of) plants. The specific relative humidities and nutrient availability experienced by the plant is established (RH between 15% and 95%; nutrient concentration as desired) during the setup of the growth environment, which takes about 5 minutes and <1$ in disposable cost. These systems maintain these environmental parameters constant for at least 14 days with minimal intervention (one minute every two days). The design is composed entirely of off-the-shelf components (e.g., LEGO® bricks) and is characterized by (i) a separation of root and shoot environment (which is physiologically relevant and facilitates imposing specific conditions on the root system, e.g., darkness), (ii) the development of the root system on a flat surface, where the root enjoys constant contact with nutrient solution and air, (iii) a compatibility with root phenotyping. We demonstrate phenotyping by characterizing root systems of Brassica rapa plants growing in different relative humidities (55%, 75%, and 95%). While most phenotypes were found to be sensitive to these environmental changes, a phenotype tightly associated with root system
topology—the size distribution of the areas encircled by roots—appeared to be remarkably and counterintuitively insensitive to humidity changes. These setups combine many of the advantages of hydroponics conditions (e.g., root phenotyping, complete control over nutrient composition, scalability) and soil conditions (e.g., aeration of roots, shading of roots), while being comparable in cost and setup time to Magenta® boxes.

Introduction

We are interested in understanding the role of environmental factors in the development of plants and ecosystems. Our initial effort focuses on developing laboratory scale growth environments that control and monitor the environment of individual plants in space and time (e.g., humidity, water availability, nutrient availability) during their growth. This capability is currently not possible in the field and is beyond the common protocols and infrastructures of laboratories (e.g., growth chambers).

We describe in this paper an experimental system that provides self-contained, sterile, growth environments for individual plants that are programmable to control (for at least 14 days) constant relative humidity (RH, between 15% and 95%) and homogenous nutrient availability. In these environments, the root system develops onto a flat sheet of paper that is saturated with the nutrient solution. The seed is sowed into a plug that is lodged into a plastic sheet that separates the environment of the root from that of the shoot. The separation between the root and the shoot environment is important because (i) it reduces the evaporation from the nutrient reservoir, which eliminates nutrient accumulation and enables an effective control of humidity at the shoot, (ii) it facilitates the shading of the root system from light (cf. S10 Fig), and (iii) it is more similar to the physiological growth conditions of the plant. An earlier design of this approach achieved a homeostatic control of humidity through the use of saturated salt solutions, but could not limit the accumulation of nutrients in contact with the roots due
to evaporation of the nutrient solution[1]. Furthermore, the range of attainable relative humidities was limited between ~50% and 95% and therefore could not simulate truly desiccating conditions.

Growth chambers or phytotrons for individual (or few) plants provide several advantages over larger scale equipment (e.g., large growth chambers) or facilities (e.g., greenhouses). Environmental control. Because of the historical emphasis on studying and breeding plants in loosely defined “physiological” environments, the current infrastructure and methods for plant science and breeding are very sophisticated when it comes to plant characterization (e.g., confocal microscope, Genome-wide association studies), but less so when it comes to plant growth. Humidity, for example is a very difficult parameter to control, especially at scale [2–5]. Other parameters (e.g., nutrient composition, heterogeneities such as nutrient gradients) are difficult to control in time and space (especially in field trials) since they are dependent on the type of "soil" media the plants are growing in [6, 7]. Controlling environments is easier in small volumes than it is in large volumes (think, for example, about sterile conditions): our environments maintain constant humidity and nutrient concentration in sterile conditions without requiring electrical power. New data. Standardized, self-contained, highly modular, and customizable plant environments enable unique experiments based on exposing plants to unique environmental stimuli. Many of the most interesting questions with respect to plant development relate to how local environmental cues lead to a global phenotype. Individual stress testing. Due to the ineffectiveness of growth chamber/greenhouse environments at testing plants' responses to the environment, the bulk of the "stress-testing" of plants in breeding is performed in field trials. These pipelines are expensive and slow and have a low success rate [8, 9] also because stress intolerant plants were not removed from the candidate pool at the greenhouse stage. It is therefore useful to develop systems that grow individual (or small groups of plants) plants with a better control of environmental conditions for laboratory scale experiments as well as large phenotyping trials. Individual plant environments would allow stress testing on a select number of plants in laboratories. Logistics. Individual,
self-contained growth environments would enable the plant science experiments without requiring dedicated, expensive growth facilities (rhizotrons, growth chambers, greenhouses) that may not be available to researchers from other disciplines. *Reproducibility*. The lack of universally embraced standards in plant growth protocols considerably reduces reproducibility[10]. Despite internal controls, many environmental variables are almost never rigorously controlled for (e.g., biotic environment of plants, light quality). The development of integrated, standardized tools for controlling the environment surrounding individual plants would enable improvements in experimental reproducibility that are necessary to address complex biological questions such as Genome-by-Environment (GxE) effects. *Failure tolerance*. Single plant environments, because they are confined and distributed, limit and contain failure (e.g. due to disease or contamination), thereby reducing the risk of catastrophic experiment loss. *Robustness*. Because of their untethered, simple design, single plant environments are less likely to break, to malfunction, to degrade. *Higher data quality*. Single plant chambers with accurate environmental control could reduce experimental variability and therefore enable the design of experiments that reduce replicate numbers in favor of highly controlled environmental conditions with low failure rates. Data quality and highly controlled experiments is an approach to bring value to small laboratory operations to complement large facilities.

The plant/soil/environment system is a complex, highly correlated system. There are two main approaches to studying such systems: a holistic approach, preferably data-intensive, in which the real system is monitored in its full complexity and where analysis of the data can bring out correlations, suggest hypotheses, and sometimes make predictions [11, 12]. The other is a reductionist approach that produces model systems in which a select number of variables (typically very few) can be independently changed and monitored, therefore enabling the systematic testing of hypotheses[12, 13].

The first approach is increasingly common in plant science, as shown by the use of sophisticated characterization techniques for phenotyping in facilities [14–18] and in
the field [19, 20], with the intent to produce higher quality and quantity of data for predictive phenotyping. The second approach is also very common in plant science but is mostly focused on *organismal* model systems (e.g., Arabidopsis thaliana, Populus trichocarpa) rather than *environmental* model systems (e.g., Petri dishes, Magenta boxes, phytotrons), which have not substantially improved over the past decade. While these very simple environmental model systems have been invaluable in developing knowledge, and useful in formulating and rapidly testing hypotheses [21–24] they do not provide a close enough model of field conditions (leading, for example, to a frustrating lack of correlation between lab performance and field performance of plants), and they cannot adequately provide reproducibility across labs and field conditions [10, 25]. With the help of the engineering toolbox, environmental model systems can be designed to rigorously, robustly control previously challenging or inaccessible environmental variables (e.g., chemical gradients, microbiome), while remaining simple, cheap, scalable, reusable, modular, and easy to use [1, 26].

**Experimental Design**

Plants are systems out of equilibrium which drive change in their environment by moving mass and energy and reacting chemicals. Therefore, it is challenging to create simple systems that establish a programmed steady state and that, at the same time, fulfill a long list of design constraints associated with experimental plant science. For a growth environment to be useful for plant studies it should be scalable (and therefore inexpensive and untethered from electrical power), simple to assemble, chemically inert, autoclavable, transparent, and relying on off-the-shelf components.

We wish our systems to be operated outside of sterile environments, e.g., on a laboratory benchtop. Therefore we opted for a fully enclosed system that can be easily and rapidly (5 min) assembled in a biosafety cabinet (cf. S1 Movie) and then placed anywhere. The outside enclosure should be transparent for illumination and we used a commercially available polypropylene box (Sterilite® brand).
Separate, dedicated, germination environments are useful because they allow to select similarly developed plants as replicates for experiments in the growth environments. We desired our germination environment to be as similar as possible (so as not to require an unnecessary number of different parts) and that would allow us to transfer the germinated seeds to the growth environment in a rapid (<1min) and simple manner (cf. S2 Movie). The germination and growth environments are shown in Fig 1A and 1B, respectively (the outer enclosure is omitted for clarity). Corresponding exploded views of the setups are shown in Fig 1C, highlighting the similarities between the two setups.

Fig 1. Germination and growth environments. Pictures and exploded views (external enclosures omitted for clarity). a) Side view of the germination setup. b) Side view of the growth setup with a Brassica rapa plant. c) Exploded views, to scale, of the germination (left) and growth (right) environments (units of length are mm).
In the germination environment (Fig 1A), a plastic cup is used to hold nutrient solution. A perforated plastic sheet is suspended horizontally in the nutrient solution with the help of transparent (i.e., polycarbonate) LEGO® bricks. Seeds of the plant to be germinated are sowed into a gel (0.5% agar) held by pipette tips, which are then lodged into the perforations of the plastic sheet until their bottoms dip into the nutrient solution. The seeds germinate in the plug and the roots grow out of the holes at the bottom into the hydroponic solution. This hydroponic geometry greatly simplifies the handling of large numbers of seeds and the maintenance of the system. The use of plugs (i.e., cut pipette tips) to hold the seeds enables the rapid transplantation of the germinated seedling to the growth environment.

The growth environment differs from the germination environment only by a few components. A pad of paper (Whatman #1 filter paper or blotting paper) is placed above the perforated plastic sheet and is nearly fully immersed in the nutrient solution. On top of the pad is a single sheet of paper (the “growth” sheet, Whatman #1 filter paper). The growth sheet wicks water and nutrients from the saturated paper pad. On the four corners of the growth sheet are four silicone rubber spacers that support a polycarbonate sheet with a hole in its middle. The seed plug started in the germination environment is placed in this hole. The top plastic sheet is fitted with a port for drawing and introducing liquids into the nutrient cup and the whole system is wrapped by plastic wrap. This closed environment is then placed into the outer enclosure surrounded by salt that establish the desired humidity in the environment of the shoot.

The setups are entirely reusable, with the exception of the paper pad and growth sheet. The salt can be dried in a rotary evaporator or an oven. The cost of the setup shown is <8$, while the cost per experiment is <1$ even with the cost of the seed. The setup can be easily scaled and its capabilities are conserved as long as these essential characteristics are preserved: (i) a short distance (<3 mm) between the level of the nutrient solution and the growth sheet, (ii) a paper pad with a thickness equal or greater than the typical separation between the holes in the perforated sheet, (iii) a
proper seal of the nutrient cup with plastic wrap (or analogous method) to limit evaporation of the nutrient solution, (iv) a port to replenish the nutrient cup as necessary.

The seedlings transplanted from the germination environment develop their roots onto the growth sheet, remaining in constant contact with both their nutrient and water supply as well as air. This approach allows us to combine the advantages of hydroponics (e.g., tight control over nutrient availability) and particulate systems (e.g., root access to oxygen) at the expense of the three-dimensionality of the root system. 2D root systems are very common in the study of roots by the use of rhizotrons or rhizoslides. The main differences between our approach and rhizotrons are that the growth sheet in this system is held horizontal, and that the roots are exposed to air. As it will be shown later, growth on flat surface tends to produce a more entangled but also more symmetric root system that could facilitate the detection of weak tropisms and root development responses.

Results and Discussion

Establishment of a programmed steady state of nutrient concentrations and humidities requires an understanding of the mass flows into the system caused by evaporation and transpiration (Fig 2A). The water cycle in the system is fairly simple. Water from the nutrient cup is wicked by the paper pad and the growth sheet from which it evaporates into the root environment. Since the root environment is a closed system the humidity reaches rapidly 100%, leading to condensation. Some leaks lead to a net water loss from the root environment into the shoot environment through evaporation ($J_{\text{evap}}$). As it will be shown, the design tolerates minor leaks without compromising the control over RH and nutrient concentrations. Evaporation of the agar in the seed plug is prevented by sealing the agar in the plug with wax (this step is essential to prevent the drying of the agar in the first day after transplantation). Water is also extracted from the nutrient cup by the root system and the majority of it is then transpired by the leaves in the shoot environment ($J_{\text{transp}}$) while the remainder (usually less than 1%[27]) is stored in plant
tissues. The shoot environment is a closed environment: in the absence of water sinks, the humidity reaches rapidly 100%. In our setup, hygroscopic salt (e.g., NaCl) is added on the outside of the nutrient cup and acts as a water sink. The adsorption of the water by the salt \( J_{ads} \) will, at steady state, match the combined flow of water from evaporation and transpiration \( J_{evap,H_2O} + J_{transp,H_2O} \), and establish a steady state RH. The value of the RH at steady state will depend on the composition of the salt (any supersaturated solution establishes a certain vapor pressure of water at equilibrium[28]) and on kinetics. If the rate at which water vapor is introduced in the shoot environment is larger than the maximum rate at which the salt can absorb it (which will depend, in first approximation, on the area of the exposed supersaturated solution), then the average relative humidity established at steady state will be larger than the one predicted by equilibrium thermodynamics in a closed system. These kinetic limitations were the key issue with the previous design in which the growth sheet was exposed to the shoot environment, therefore yielding a very large \( J_{evap,H_2O} \), especially for low humidities: LiCl, which establishes a RH of \(~11\%\) at room temperature at equilibrium was only able to reduce the humidity of the environment to \(~50\%\). The homeostatic regulation of RH, of course, persists only as long as the salt forms a supersaturated solution. After the salt has completely dissolved, the RH will gradually increase.

The steady state rate of water loss from the nutrient cup will be \( J_{evap,H_2O} + J_{transp,H_2O} \). This rate will be matched exactly by \( J_{ads,H_2O} \) leading to a constant concentration of water vapor in the shoot environment and a constant RH.

In our system the total water loss from the nutrient cup into the shoot environment \( (J_{evap,H_2O} + J_{transp,H_2O} = 4+1 \text{ ml/day}) \) was low enough that the RH in the shoot environment (measured through port 2 cm above height of plastic sheet) is close to the equilibrium value (from \(~15\%\) with LiCl to \(~95\%\) with Na\(_2\)SO\(_4\)). Fig 2B shows the observed RH (\( n = 2 \)) in the shoot environment (blue) as a function of the salt used, compared to the expected equilibrium RH (black). The small discrepancy between observed and equilibrium values is consistent with minor leaks in the external enclosure and with the
Fig 2. Mass flows in the growth environment and humidity control. a) Schematic of the water flows (blue arrows) and nutrient flows (red arrows) in the growth environment. On the side is a depiction of the nutrient concentration gradient formed in the part of the paper support that is exposed to evaporation. b) Observed relative humidities measured in the shoot environment, compared to the equilibrium values for a number of different supersaturated salt solutions. Error bars are 95% confidence intervals, n = 2. c) Observed relative humidities as a function of time for systems without (filled symbols) and with a plant of Brassica rapa plants (open symbols), compared to the equilibrium values at 20°C (dotted lines). Error bars are 95% confidence intervals, n = 15.
exchange of water vapour with the laboratory environment, whose humidity is generally around 50%. Since our systems provide a sterile environment over at least 3 weeks, we attribute the leaks to the specific (and apparently imperfect) modifications (a ~3 cm hole in the top) we had to implement on the external enclosure to fit a hygrometer.

The programmed steady state RH was preserved for over three weeks (Fig 2C) and was maintained even in the presence of a plant for at least two weeks (Brassica rapa’s root system would outgrow the system after that). The data in Fig 2C show the RH observed (n = 15) in the shoot environments as a function of time and salt, with (open symbols) and without (filled symbols) a plant. We were not successful in transplanting a plant into the 15% humidity environment produced by LiCl probably due to severe transpiration stress added onto the transplantation shock. Methods for changing the RH over time will be the subject of future work. Future work will also provide control over other environmental parameters such as temperature and aeration which cannot be currently controlled independently from relative humidity and nutrient concentration. With the current design, temperatures inside the system are usually one degree Celsius above the ambient room temperature and aeration relies on the diffusion of CO2 through the Parafilm seal in the outer container, which is, at the moment insufficient to maintain stationary CO2 levels for mature plants.

The transport of nutrients is connected with the transport of water and adsorption to surfaces. As water evaporates from the root environment, nutrients concentrate on the growth sheet (at a rate $J_{\text{evap, nutrients}} = J_{\text{evap,H}_2\text{O}}*[C] *FW/0.01$, where [C] is the molarity of the nutrient in mol/l, FW is the formula weight in g/mol). Transpiration also drives nutrients to the growth sheet ($J_{\text{transp,nutrients}}$), some of which will be absorbed by the plant ($J_{\text{ads,nutrients}}$). Accumulation of nutrients on the growth sheet due to water transport in the system will establish a gradient of concentration of nutrients which will drive a flow of nutrients ($J_{\text{diff}}$) from the growth sheet back into the bulk nutrient solution. We can overestimate the expected accumulation of nutrients at the growth sheet by making the following assumptions. We approximate that the concentration of
nutrients throughout the bulk of the nutrient solution is constant \( (C_{\text{cup}}) \). The distance between the surface of the nutrient solution and the surface of the growth sheet, \( h \), is typically 1mm but can be overestimated at 2mm. We neglect \( J_{\text{ads,nutrients}} \), thereby assuming that all nutrients brought to the growth sheet by \( J_{\text{evap,H}_2\text{O}} + J_{\text{transp,H}_2\text{O}} \) accumulate on the growth sheet. In our experiments \( J_{\text{evap,H}_2\text{O}} + J_{\text{transp,H}_2\text{O}} = 0.05 \) ml/cm\(^2\)-day, which, for phosphate, corresponds to \( J_{\text{evap,nutrients}} + J_{\text{transp,nutrients}} = 0.001 \) mg/cm\(^2\)-day. At steady state, this flow of nutrients is matched by the downward flow of nutrients \( (J_{\text{diff}}) \) driven by the difference \( \Delta C \) in the concentration of phosphate between the top of the growth sheet \( C_{\text{growth sheet}} \) and the nutrient cup \( C_{\text{cup}} \). Using a value of diffusivity of \( 0.89 \times 10^{-5} \) cm\(^2\)/s \([29]\) and solving \( J_{\text{diff}} = D \cdot \Delta C / h \) for \( \Delta C \) gives an estimated steady state concentration of nutrients at the growth sheet that is only 1.3\% higher than that in the nutrient cup. Nutrients can also adsorb onto surfaces and become unavailable to the plant. In our system the nutrient solution contains a rather large amount of paper that can coordinate ions. It is important to compare the concentration of nutrients in the bulk liquid and compare it to the concentration introduced into the system.

Fig 3A shows the concentration of essential nutrients in the nutrient cup (open symbols and dashed lines, \( n = 8 \)) as well as on the growth sheet (filled symbols, \( n = 8 \)) during the growth of a plant \( (Brassica rapa) \) for about two weeks.

The data indicates that (i) there is no nutrient accumulation for about 2 weeks of plant growth (the concentrations in the cup are not significantly different from those observed on the growth sheet), and that (ii) the large paper pad does not immobilize a significant fraction of the nutrients in the nutrient solution. The moderate decrease in the nutrient concentration can be attributed to plant uptake, since the liquid level in the nutrient cup was always reestablished with DI water (i.e., there was no input of nutrients in the system throughout the experiment).
The flow of nutrients in the system is not only limited to the vertical axis but also occurs horizontally. Any heterogeneity in the horizontal distribution of nutrients on the growth sheet would result in an uneven distribution of nutrients across the root system of the plant, thereby driving chemotropic root development. The overall point to point concentration heterogeneity (one standard deviation) in our system was 11%. Fig 3B shows the average deviation from the average growth sheet nutrient concentration of the points of the growth sheet located close to the roots (<5mm) versus those located far from it (>5 mm), and shows that there is no significant difference between the two (p = 0.83). This data indicates that the adsorption of nutrients from the growth sheet does not lead to a significant nutrient depletion or accumulation in proximity of the root. The result is meaningful especially when comparing it with the nutrient depletion observed around the root systems grown on gels and other media[30].

The platform is compatible with root phenotyping, albeit not in situ. The stem must be severed to expose the root system. Fig 4A shows the comparison of the root systems of two *Brassica rapa* plants grown in 95% and 55% RH, respectively.

The biomass of the root and shoot (Fig 4B) depends on the humidity experienced by the shoot (p = 0.02 and p = 0.03 for a significant difference between 55% and 95% RH for root and shoot biomass respectively), while the ratio between the biomass of the root and shoot did not change significantly. The biomass information is closely correlated to root phenotypes obtained through image analysis of photographs of the root system, e.g., root surface area, root span (calculated as half of the maximum width of the root system). For example, the Pearson product-moment correlation coefficient between root span and root biomass is 0.9996, while it is 0.96 between root span and shoot biomass. This finding suggests that simple analysis of root system photographs can yield—with prior calibration—biomass information even for highly overlapped root systems grown on a flat surface.
Fig 3. Nutrient concentrations in the growth environments. a) Concentrations of essential nutrients measured in the nutrient cup (filled symbols) and on the growth sheet (open symbols), as a function of time, in the presence of growing Brassica rapa plants (error bars are 95% confidence intervals, n = 48). Dotted lines indicate the initial concentration of nutrients (0.5 Murashige and Skoog, MS) b) Deviation from average nutrient concentration in regions proximal to the root (5 mm), (error bars are 95% confidence intervals, n = 24)

The ratio between maximum perpendicular dimensions of the root system (“root symmetry” phenotype, Fig 4B) indicates that the root system is highly symmetric in our growth environments, thereby supporting the possibility of studying quantitatively weak tropisms by quantifying asymmetry of the root system.
Fig 4. Root phenotyping. a) Representative thresholded images of root systems of Brassica rapa grown in 55% RH (left) and 95% RH (right). b) phenotypes as a function of RH (55%, n = 15; 75%, n = 19, 95%, n = 17): root biomass (circles), shoot biomass (squares), and root/shoot biomass ratio (up triangles) as compared to the surface area (down triangles), the span (rhombi), and the symmetry (stars) of the root system. The lines between scatters are guides to the eye. The lines above and below the scatters identify 95% confidence intervals. c) Frequency of the sizes of areas on the growth paper that were fully enclosed by roots of Brassica rapa plants grown in 55%, 75%, and 95% RH.

Root systems are generally considered to be extremely plastic to their environment[31]. While phenotypes that strongly respond to environmental conditions
are useful for studying and optimizing GxE interactions, phenotypes that are robust towards environmental parameters (albeit rare) can be also useful in assessing phenotypic changes induced purely by the genotype. Fig 4C shows a root architecture phenotype that displays a remarkable robustness against relative humidity changes. Analysis of the thresholded root photographs allowed us to extract the areas (in cm²) that were fully enclosed by roots. The distribution of these areas is shown in Fig 4C for all sets of plants, in a log-log plot. The coincidence between the distributions is very striking, especially considering that the roots had to be transferred to a black support before their imaging, and that the thresholding process was not flawless (e.g., the distribution is likely truncated at large areas because their large perimeters make them especially subject to imperfect thresholding). The relatively linear trend on a log-log plot indicates the possibility that the void areas follow a power-law scaling that is characteristic of self-similar and fractal structures[32].

**Conclusion**

We have shown a practical approach to the germination and growth of seedlings in nearly homeostatic conditions of relative humidity (between 15% and 95%) and nutrient concentrations. The setups are completely self-contained, untethered, and create two separate environments for the root and for the shoot. The root system develops on a moist, flat sheet of paper, in ~100% RH, but in constant contact with air. The shoot develops in an environment whose humidity is determined by a supersaturated salt solution. The initial conditions of their assembly are used to program the RH and nutrient concentrations that the plant will experience for 2–3 weeks. The nutrient concentrations are found to not change substantially over the course of two weeks, with minimal spatial variations, regardless of the proximity of a plant root. The general design can be easily scaled to larger plants and can be modified to allow for different environmental conditions (e.g., shading of the root). The specific setups reported here cost <8$ (the cost per experiment is <1$ including the cost of the seed), and can be assembled in 5 min.
Materials and Methods

Determination of Concentration Gradient

The concentration gradient in our system at steady-state can be determined using the 1-D solution to Fick’s 1st law of diffusion

\[ J = -D \frac{\Delta [C]}{\Delta z} \]

Where

- \( J \) is the diffusive flux expressed in units of \( \frac{mol}{cm^2 \cdot s} \)
- \( D \) is the diffusion coefficient in units of \( \frac{cm^2}{s} \)
- \( \Delta [C] \) is the concentration gradient in units of \( \frac{mol}{cm^3} \)
- \( \Delta z \) is the distance over which the flux occurs in units of \( cm \)

The flux is first determined by considering the water loss from the nutrient cup. This flux is the combined result of flow of water from evaporation and transpiration (\( J_{evap,H2O} + J_{transp,H2O} \)). In our case, the average result was \( J_{evap,H2O} + J_{transp,H2O} = 4+1 \text{ ml} / 100 \text{ cm}^2 \text{ paper} \cdot \text{day} \) for each system. These fluxes together we will define here \( J_{H2O} \) which can then be equated to \( J_{evap,nutrients} \). \( J_{evap,nutrients} = J_{H2O} \cdot [C] \cdot FW / 1000 \), where \([C]\) is the molarity of the nutrient in mol/l, FW is the formula weight in g/mol). \( J_{evap,nutrients} \) is directly matched by the downward flow of nutrients which we call \( J_{diff} \). From \( J_{evap,nutrients} \) or \( J_{diff} \) the concentration gradient is determined with the known diffusivity of the ion of interest[1] and the initial concentration of the ion in the nutrient cup. An estimate of each ions accumulation at steady state can then be determined.

Sample calculation for Phosphorus at steady state

\[ J_{H2O} = J_{evap,H2O} + J_{transp,H2O} = \frac{1 + 4 \text{ ml} H_2O \text{ day}}{100 \text{ cm}^2 \text{ paper}} = 0.05 \frac{\text{ml} H_2O}{\text{cm}^2 \cdot \text{day}} \]

\[ = 5 \times 10^{-5} \frac{L H_2O}{\text{cm}^2 \cdot \text{day}} \]
mass flow of $P = \frac{20.45 \, mg \, P}{L} * \frac{1 \, g \, P}{1000 \, mg \, P} * \frac{1 \, mol \, P}{30.97 \, g \, P} = 0.0066 \, \frac{mol \, P}{L}$

$J_{evap, P} = 5E^{-5} \frac{L \, H_2O}{cm^2 \cdot day} * 0.0066 \, \frac{mol \, P}{L} * \frac{30.97 \, g \, P}{1 \, mol \, P} = 1.02E^{-6} \, mg \, P/cm^2 \cdot day$

$J_{evap, P} = -D \frac{\Delta [C]}{\Delta z}$

$J_{evap, P} = 1.02E^{-6} \, mg \, \frac{P}{cm^2 \cdot day} = - \frac{0.89E^{-5} \, cm^2}{s} * \frac{\Delta [C]}{0.2 \, cm}$

Solve for $\Delta [C]$:

$\Delta [C] = 0.0026 \, \frac{mg \, P}{cm^3} = 0.27 \, \frac{mg}{L}$

Using the initial $P$ concentration of nutrient cup and the calculated $\Delta [C]$ determine the estimated accumulation of $P$ at steady state:

$\% \, accumulation \, P$

$= \left( 20.45 \, \frac{mg}{L} + 0.27 \, \frac{mg}{L} \right) - 20.45 \, \frac{mg}{L} / 20.45 \, \frac{mg}{L} * 100\% = 1.3\%$

Variance calculations

The variance of from spot to spot on the paper growth sheet was determined for all ions. This calculation is based on the the average variance of all ions. The variance of ions was also determined based on the proximity to the root, i.e. away from the root or near the root. Away from the root was only considered if it was at least 5 mm away from any root in any direction. 6 spots where sampled on each pad of each system. The Root mean square (RMS) was determined for all systems used in the plant study to give indication of the error of concentration from spot to spot.

The relative variance of nutrients on the growth sheet considers each spot with the corresponding pad system using the following equation:

$relative \, variance \, of \, each \, spot = \frac{[ion \, concentration \, at \, spot] - [average \, ion \, concentration \, over \, that \, pad]}{[average \, ion \, concentration \, over \, that \, pad] * 100\%}$
Example Calculation for Whatman system 3

\[ \text{average } NH_4^+ \text{ concentration on pad} = 126.58 \text{ ppm} \]

\[ NH_4^+ \text{ concentration at spot } 1 = 126.25 \text{ ppm} \]

\[ \text{relative variance of } NH_4^+ \text{ at spot } 1 = \frac{[126.25 \text{ ppm}] - [126.58 \text{ ppm}]}{[126.58 \text{ ppm}]} \times 100\% \]

\[ = -0.2607\% \]

An average variance was determined for the proximity away from the root over all systems by averaging all relative variances of spots of all systems that were at least 5 mm away from any root. The average relative variance was also determined for all spots near the root for all systems. The average relative variance near and away from the root is tabulated below.

<table>
<thead>
<tr>
<th>Spot proximity to root</th>
<th>Average Relative Variance</th>
<th>Standard Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Away from root</td>
<td>0.2938 %</td>
<td>0.0109</td>
</tr>
<tr>
<td>Near root</td>
<td>-0.2908 %</td>
<td>0.0192</td>
</tr>
</tbody>
</table>

The root mean square (RMS) was determined for all spots for all ions across all pad systems. The square mean of each ion's relative variance was determined by the following equations

\[ \text{Mean}^2_{NH_4^+} = ((\text{relative variance of } NH_4^+_{\text{spot } 1, \text{ system } 1})^2 + (\text{relative variance of } NH_4^+_{\text{spot } 2, \text{ system } 1})^2 + \cdots + (\text{relative variance of } NH_4^+_{\text{spot } n, \text{ system } n})^2 \]
Mean$^2_{NO_3^-} = \left((\text{relative variance of } NO_3^-_{\text{spot } 1, \text{ system } 1}\right)^2$
+ \left((\text{relative variance of } NO_3^-_{\text{spot } 2, \text{ system } 1}\right)^2 + \ldots$
+ \left((\text{relative variance of } NO_3^-_{\text{spot } n, \text{ system } n}\right)^2$

Mean$^2_{P^+} = \left((\text{relative variance of } P^+_{\text{spot } 1, \text{ system } 1}\right)^2$
+ \left((\text{relative variance of } P^+_{\text{spot } 2, \text{ system } 1}\right)^2 + \ldots$
+ \left((\text{relative variance of } P^+_{\text{spot } n, \text{ system } n}\right)^2$

Mean$^2_{K^+} = \left((\text{relative variance of } K^+_{\text{spot } 1, \text{ system } 1}\right)^2$
+ \left((\text{relative variance of } K^+_{\text{spot } 2, \text{ system } 1}\right)^2 + \ldots$
+ \left((\text{relative variance of } K^+_{\text{spot } n, \text{ system } n}\right)^2$

The RMS was determined by

\[ RMS = \sqrt{\frac{1}{n} (\text{Mean}^2_{NH_4^+} + \text{Mean}^2_{NO_3^-} + \text{Mean}^2_{P^+} + \text{Mean}^2_{K^+})} \]

Where $n$ is the sum of all relative variances for all ions in the study

<table>
<thead>
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<th>Ion</th>
<th>$n$</th>
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<th>RMS</th>
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<tr>
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</tr>
<tr>
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<tr>
<td>P$^+$</td>
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<tr>
<td>K$^+$</td>
<td>160</td>
<td>284.949 %</td>
<td></td>
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<td>All ions</td>
<td>656</td>
<td>91863.926 %</td>
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References


CHAPTER 7. FINDING WATER- USING WAX PAPER PRINTED PAPER AS METHOD TO STUDY ROOT HYDROTROPISM

Kara R. Lind, 1 Oskar Siemianowski 1, Bin Yuan 2, Tom Sizmur 4, Ludovico Cademartiri 1,2,4 *

1 Department of Materials Science & Engineering, Iowa State University of Science and Technology, Ames, IA
2 Department of Chemical & Biological Engineering, Iowa State University of Science and Technology, Sweeney Hall, Ames, IA
3 Soil Research Centre, Department of Geography and Environmental Science, University of Reading, Whiteknights, Reading RG6 6DW, UK
4 Ames Laboratory, U.S. Department of Energy, Ames, IA

Abstract

Water is the most significant limiting factor for crop yields. Therefore, understanding the hydrotropic response in plants (i.e., the development of roots in response to an inhomogeneous water distribution) is important to breed or design genotypes that potentially make more efficient use of a limited water supply (e.g., due to drought or to soils with low water retention). A number of studies explored the mechanism behind hydrotropism in single root tips by using osmolytes in gels and moisture in air assays, but the quantitative exploration of changes in the architecture of whole root system as a result of a known, inhomogeneous, water distribution is deceptively challenging. As far as we know, there are no technical means to generate stationary and arbitrary water distributions across a surface for the high throughput phenotyping of hydrotropic tendencies of whole root systems.

In this paper we describe a simple methodology to grow roots on a paper microfluidic device by which we control the overall amount of water available to the plant as well as where it is located with respect to the seed with a 0.83 mm spatial resolution.

We use this methodology to show that, as water becomes more rare, plants of Brassica rapa concentrate a more significant fraction of their root system where water is available (up to 15% when only 1% of the available area has water, as opposed to 2% in
the control). The lack of evidence for increased branching at the water sources suggest that this phenotype is purely the result of a hydrotropic, water-seeking response.

**Introduction**

[The roots grow on a filter paper that has been printed with patterns of wax by a commercial printer. The paper is fed with nutrient solution from a reservoir by capillarity, but the areas printed with wax remain dry. Since the printed wax is stationary, the water distribution is constant in time.]

We, and others [1-6], are interested in understanding the influence of an inhomogeneous water supply on the development of plant root systems. Unless the efficiency with which crops use water is improved, water consumption in agriculture will need to increase 20% by 2050 to meet demand for food[7], even though the irrigation of crops already accounts for ~70% of global fresh water use[8] and two thirds of the world population are projected to be in conditions of water scarcity by 2050[9]. These constrains are further worsened by the expected impact of climate change[10-12].

Roots respond to moisture gradients by bending towards areas of higher water availability through a physiological process called hydrotropism. Knight characterized hydrotropism in 1811[13]. Since 1872, hydrotropism was reinvestigated by Sachs[14], Molisch[15], Darwin[16], and, more recently, by others[5, 17-20]. Quantitative studies of root hydrotropism inside the laboratory are very challenging. In this work, we will present a laboratory scale growth environment that relies on wax paper printing to overcome some of these difficulties. This approach can be used by non-experts in any location, worldwide, to design and perform novel experiments related to hydrotropism and drought. Printing is rapid, versatile, low cost and simple. Patterns can be designed,
printed and used in experiments, by non-experts, in minutes[21, 22]. The patterns that can be printed are limited mainly by the creativity of the researcher. Paper is also a very versatile material. Paper can be cut, folded, stacked or wrapped around objects to, potentially, create custom environments that provide 3-dimensional control over water availability[23].

This approach like others used for hydrotropism[24] is cheap (<$1 per replicate) however, it offers several advantages over other platforms. Generating an arbitrary and stationary spatial distribution of water availability across a cm-scale root system is not currently possible by any technical means, as far as we know. Gradients of water availability can be established by creating a water potential gradient in a gels with osmolytes (e.g. glycerol[25] or sorbitol[26]) which induces an asymmetric water deficit in the plant tissues (i.e. split agar assay). A water availability gradient can also be established by creating a humidity gradient in air[27] (i.e. moisture in air assay). While both of these approaches lead to many findings related to hydrotropism, neither is able to establish stationary and arbitrary moisture gradients that can be combined with high throughput phenotyping of whole roots systems. Instead, they limit studies on hydrotropism to singular root response (i.e. turning of the tap roots).

Root systems respond to other stimuli (e.g., gravity, oxygen, chemicals, temperature, light, touch). Isolating hydrotropism from the other “tropisms” can be challenging. Hydrotropic responses could be exaggerated or biased due to a lack of surface (i.e. thigmotropism [28]), “toxic” chemical interactions (i.e. chemotropism) or roots interacting with light (i.e. phototropism). In particular, gravitropism is especially
challenging[29, 30] to separate from hydrotropism. Hydrotropism in most cases is
masked by gravitropism[31]. Hydrotropism can be distinguished from gravitropism by
using agravitropic mutants[32], or by growing plants in clinostats[29, 33] or
microgravity[29]. These approaches require the use of mutants (which are not available
for most species, and might not be fully representative of their species), or employ
complex, expensive, and low-throughput experimental setups. By employing the wax
paper on a flat surface where the roots can develop, we can prevent the gravitational
bias on the root development.

We here present a simple solution to growing plants under controlled and
stationary water distributions on a flat surface (Figure 1) using wax paper microfluidics.
*Brassica rapa* plants germinate and grow on an horizontal and flat sheet of filter paper
that can be printed with wax to create pores accessible to water and nutrients.

**Experimental Design**

The experimental design aimed at controlling abiotic environmental variables
and limiting their influence on the hydrotropic root response across the entire root
system.

*Choice of plant model system.* We used *Brassica rapa* (Wisconsin Fast Plants) as
a model system for its fast growth and taprooted root system.

*Limiting the influence of gravitropism, chemotropism, phototropism, and
thigmotropism on the direction of root growth.* Root development is strongly affected
by gravity[34, 35]. This influence is different for different parts of the root system (e.g.,
primary roots are strongly gravitropic, i.e., they grow perpendicularly to the
gravitational field gradient, while secondary roots are plagiotropic, i.e., grow at an angle with respect to the gravitational field gradient). Therefore, we constrained the development of roots to an horizontal surface (Figure 1A-B) to isolate the effect of hydrotropism on the development of the entire root system. While the biological process of hydrotropism remains active, it is limited in its ability to govern the direction of root growth.

Root development is also strongly affected by gradients in nutrient and chemicals concentrations by a process called “chemotropism”[36]. Since nutrients are tightly associated with water, we could only ensure that the concentration of the nutrient solution accessible to the plant would be constant in time (we used Murashige-Skoog (MS) medium at 0.5X concentration for all experiments), by leveraging an experimental setup developed previously for this purpose[37]. In summary the plant is grown on a horizontal sheet of filter paper that is imbued with nutrient solution by capillary action from an underlying reservoir (Figure 1A-B). As the distance between the reservoir and the growth surface is minimal, the concentration of nutrients in the nutrient solution contained in the growth sheet is not significantly different from the one in the reservoir and is constant in time since the absolute amount of nutrients in the reservoir is much larger than the amount of nutrients that is consumed by the plant. Lastly, to avoid the potential influence of biologically generated chemicals (e.g., by fungal or bacterial contamination of the growth surface), we enclosed the plant growth environment in a sealed, fully autoclavable container.

The roots are also significantly affected by exposure to light[38, 39]. Light affects
the direction of root growth as well as the cellular development of the root tissue. To avoid this influence we covered the root system with a slanted aluminum foil. The choice of aluminum was dictated by the low cost, facile use, and by its hydrophilic surface. As the aluminum encapsulates the root environment, condensation can easily happen on the aluminum surface facing the root. This condensation can lead to water droplets beading and dropping from the aluminum surface on the root system, thereby changing the distribution of water across the root system. Aluminum’s hydrophilicity minimizes the contact angle of water and allows for condensation to drain to the side, back into the reservoir (Figure 1A).

Finally, roots are very sensitive to contact with surfaces through a process called “thigmotropism”. The presence of a flat surface ensures that every root tip experiences the same type of contact with a hard surface.

**Control over the rate of evapotranspiration.** The rate of evaporation and plant transpiration is governed by the relative humidity of the atmosphere and, potentially, by water deficiency. Therefore, temperature and humidity are important parameters affecting the water status of a plant. Our laboratory was set to constant temperature through a redundant air conditioning system and ventilation. By enclosing the plant growth environment and using a saturated solution of sodium chloride, we established a homeostatic relative humidity for the plant shoots of 85.0% (SD = 0.77). Aeration with moist air was provided by a pump connected to a bubbler (Figure 1A).

**Spatial control over the distribution of water across the root system.** In our system, the roots grow on the top surface of a filter paper (Whatman 1 chromatography
paper) that is fed with nutrient solution from an underlying reservoir by capillarity. This capillary flow can be prevented by coating the paper fibers with a hydrophobic substance. We used a commercially available desktop printer (Xerox Colorqube), to print patterns of wax ink on the filter paper on which the root would grow. Applying heat to the wax-printed paper results in the spreading of molten wax and the coating of the paper fibers across the entire thickness of the paper, as previously described[22] and shown schematically in Figure 1C. We used steam autoclaving to simultaneously melt the wax and sterilize the paper. The width of a wax line (“wall”) after autoclaving (autoclaved wall width, AWW) was 1.62 mm wider than the printed wall width (PWW), indicating a constant lateral spreading of 0.81 mm (Figure 1D).

By this approach, the roots of the plant can be grown on a surface where dry areas (where the wax was printed and molten) and wet areas (where no wax was printed) can be created with precision and remain stationary over time. Figure 1E shows square patterns of molten wax used to create isolated wet areas (“pores”). As the printed sheet is placed on a wet support, the pores are filled with water (Figure 1F, the water is dyed in red for clarity). The transport of water into the pores is effective even for the smallest pores (0.4 mm², Figure 1G). The size of the pores does not affect the local water availability (i.e., the pressure required to draw water from the pores). As shown in Figure 1H, no water is sucked up by the capillary in the printed areas, while columns of water of identical height (15 mm, corresponding to a pressure of 147 Pa) are drawn from pores of different sizes.
Figure 1. System Design. a.) Schematic representation of platform b.) *Brassica rapa* plant on system c.) Schematic and real cross-sections of wax generation for pores c.) Printed widths as means to generate autoclaved widths (i.e. wax barriers) e.-g.) Images of system with variety pore sizes before and after dye absorption h.) Capillary testing for treatments i.-j.) Water access as a function of autoclaved pore size and autoclave width (i.e. wax barrier width)

Melting the wax blurs the printed patterns and limits the spatial resolution achievable by this approach (the thinnest dry area is 0.4 mm, while the thinnest wet area is 0.85 mm), but is necessary to avoid the delamination and displacement of the wax from the paper surface by the growing roots.
Since the size of the pores does not influence the water availability, we use the relative wet area (RWA, defined as the ratio between the area of all pores and the total area) to quantify the overall access to water on the growth surface.

Two parameters determine the relative wet area, RWA: the wall width and the pore width, as indicated in Figure 1C. Since these two parameters are controlled independently, the same RWA can obtained with more than one pore width. Square arrays of pores were created on a 10cm x 10cm filter paper by printing perpendicular arrays of walls of different widths (PWW), separated by different distances (PPW). The RWA and the individual pore area for each set of PWW and PPW were quantified by using image analysis (Figure 1I and 1J respectively).

**Results and Discussion**

*Reducing the Relative Water Area reduces biomass in plants.* Plants of *Brassica rapa* were grown at 24-26°C under ~140 PAR ± 10 PAR of illumination for 24 hours/day for 10 days from germination. Plants were germinated in a system previously described[37] for 5 days, after which they were transplanted in the setup shown in Figure 1A-B were they were forced to grow on growth sheets with 1%, 3%, 6%, 11%, 19% RWA (n=8,11,13,12,10, Figure 2A). 100% RWA (i.e., unprinted filter paper) was used as a control (n=20). 0% RWA (fully printed filter papers) led to near complete loss of the plants. The tap roots of the transplanted seedlings were spiraled into a “starter” pore of identical area (25 mm²) in each treatment to increase the rates of survival of the plants, especially for low RWA values. The autoclaved wall width (AWW) was kept constant (6 mm) across all treatments: the RWA was tuned purely by changing the APW (from 0.4
mm for 1% RWA, to 25 mm for 19% RWA). As a consequence, regardless of the RWA value, the roots had to cross the same distance of dry surface to reach a new source of water and nutrients.

Phenotyping (Figure 2B) was conducted at the end of the experiment after excising the stem. Photographs of the root systems were analyzed to characterize structural root phenotypes both in the dry areas as well as in the wet areas.

Compared to the control treatment (100% GWA), the biomass of both roots and shoots decreased with the RWA (consistently with water stress), while qualitatively following an exponential trend of the type

\[ \text{biomass}(RWA) = \text{biomass}(RWA=100\%) + A \cdot e^{rate \cdot RWA} \]

with a rate equal to -0.062±0.039 (Figure 2C, R²=0.817). A similar trend is observed in the dependence of the root surface area on the RWA (Figure 2D, same exponential trend with a similar rate of -0.055±0.05, R²=0.817). The root surface area was found to be approximately proportional to the total biomass (Figure 2E, R²=0.988), suggesting that the average root diameter is similar for all treatments. Two more phenotypes are conserved across treatments: the root density (Figure 2F, i.e., the ratio between the total surface area of the roots and the convex area of the root system), and the circularity of the root system (Figure 2G, quantifying the overall shape of the root system, p-value>0.05). Taken together, these phenotypes indicate that, while the RWA affects the overall biomass of the root system, it does not affect significantly the geometry of its development.
Our root phenotyping can distinguish the roots located on the pores from those located on the wax. The most relevant phenotype, which we call “water preference ratio” (WPR), quantifies the probability to find a root on a pore, normalized by the probability to find a pore. Therefore if WPR is equal to 1, then the roots position is indifferent to whether they are on a dry or wet spot. If WPR is equal to 2, then the roots are twice more likely to be found on the pores than by pure chance. If WPR is equal to 0.5, then they are half as likely to be found on a pore than by pure chance.

Figure 1H shows the WPR as a function of RWA. Two different curves are provided. In blue is shown the WPR calculated considering all pores, while in green is shown the WPR calculated excluding the central pore. In both cases the WPR increases exponentially with the decrease in RWA ([p-value<0.05]). This increase in the WPR could be explained by two mechanisms. The first hypothesis is that when a root finds a pore, it creates several branches, therefore increasing the root surface area located on the pores, compared to that on the wax. The second hypothesis is that the roots are deliberately seeking the pores. Our phenotyping shows that the roots on the pores do not show a particular propensity to branching, thereby disproving the first hypothesis. Nonetheless, assuming that the roots are hydrotropically driven by the gradient in the partial pressure of the water vapor at the edge of the pores, the second hypothesis is challenged by the fact that all treatments feature the same distance between pores, and the same nutrient concentration (and therefore the same water activity and equilibrium vapor pressure). Therefore, the gradients in water vapor on the surface of the growth sheet should not be significantly different.
Figure 2. Global Water Phenotypes. a.) Representative images of global water treatments b.) Phenotyping strategy used for root architecture c.) Dry root and shoot biomass of global water treatments d.) Surface area of global water treatments e.) Linear relationship between root surface area and root biomass f.) Convex area of global water treatments g.) Root surface density h.) Fraction of pores relationship for global water treatments
Seeker testing of radial treatments of pores.

To test whether the roots actively scout for pores, we hypothesized that, for an equal RWA, different distributions of pores should lead to different root system architectures. Two treatments were developed. Both patterns possess a circular pattern of 8 identical pores surrounding the central starter pore. Representative images of these treatments are shown in Figure 3A. Therefore both treatments have equivalent RWA (0.75% ± 0.04%). The value of RWA is chosen to ensure that roots are water stressed and rely on seeking pores. However, the distance between the ring of small pores and the “starter” pore was different (23 mm for the “short” treatment, and 40 mm for the “long” treatment, Figure 3A, left and right, respectively). Root and shoot biomass, circularity, total root surface area, and the root surface area on pores were not significantly different for the two treatments (Figure 3B). Nonetheless, the convex area was 60% larger for the long treatment compared to the short treatment (p-value=0.007, Figure 3B), showing that the confined water supply served to confine the root system.

To better understand how the inhomogeneous distribution of water changes the root architecture, we calculated the surface density of the roots as a function of the distance from the “starter” pore as determined by the following equation,

$$\text{root surface density}(r) = \frac{\text{surface area of the roots (r)}}{2\pi r \times \text{total root surface area}}$$

, where r is the distance from the “starter” pore. Roots are more dense (p-value< 0.05) between r=0-20mm in the short treatment than in the long treatment (Figure 3C).
Figure 3. Water Seeking Phenotype a.) Comparison of total root surface area, root convex area, and surface area of roots on pores for long and short treatments (***p-value<0.01, n=15, 19 for short and long treatments respectively) b.) Percentage of total roots surface area in radial slices. Solid line and dashed line represent location of short and long treatment pores respectively (*p-value<0.09, **p-value<0.05, ***p-value<0.01) c.) Representative images of long and short treatments

For longer distances, the density of the roots in the short treatment drops drastically, while that of the long treatment decreases less rapidly. The density of roots in the long treatment is significantly larger than that in the short treatment for distances of 50 mm (p-value= 0.0006) and 60 mm (p-value=0.035) (Figure 3C).

Conclusion

We described a new platform for the quantitative study of hydrotropism in whole root systems. The approach is simple and allows for the creation of arbitrary,
stationary distributions of wet and dry areas across a flat, horizontal surface on which
the root is grown and where it can be easily phenotyped. The apparatus is further
designed to shield the roots from light, control the humidity of the shoot, provide
aeration. The relative area of the wet areas was found to influence the plant biomass
consistently with water stress. Inhomogeneous distributions of water caused the plants
to develop significantly different root phenotypes that indicated a quantifiable tendency
to pursue water. We suggest that this tool could be useful for (i) studying hydrotropic
responses of whole root systems on the lab benchtop, (ii) identifying new traits and
phenotypes associated with tolerance of scarce water, and (iii) rigorously and
quantitatively comparing hydrotropic responses in different germplasms.

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CHAPTER 8. CONCLUSION FOR ENVIRONMENTS BY DESIGN

In this work, we developed a series of tools that can be employed on the lab benchtop. These tools have been designed to allow hypothesis based testing by controlling the interactions and/or environment of the plant. By hijacking commercially available plastics and papers, we can create environments that are low cost but still scalable for plants.

The first set of tools rely on the use of LEGO® bricks which can be used as building blocks for the construction of modular ecosystems for plants. Besides their modularity, LEGO® bricks are transparent to light but also compatible with sterilization procedures. Our redesign of LEGO® bricks ecosystems resulted in one of our most robust tools for plants sciences: Habitats for Organisms and Modular Ecosystems (HOMEs). HOMEs allow us to start to investigate important ecosystems such as the rhizosphere or communities of plants. The creation of networks of HOMEs housing individual organisms (from plants to microbes) allow complete control over the chemical signaling as well as independent control over each organisms environment.

The second set of tools use paper to facilitate high-throughput phenotyping of root systems. Papers saturated in nutrient solution are one remarkably simple way to provide a flat and horizontal growth surface for plant roots. Combining our paper platform with wax printed paper could allow researchers to engage in one of the most important questions in global agriculture known: How do plants sense water? We show that using this approach we can control the amount of water available to a plant by the generation of stationary and arbitrary water distributions. We can use these water distributions to uncover important water seeking phenotypes of roots.