Video Article

A Flexible Low Cost Hydroponic System for Assessing Plant Responses to Small Molecules in Sterile Conditions

Carolina C. Monte-Bello*[1,2], Elias F. Araujo*[1,3], Marina C.M. Martins[1], Valeria Mafra[4], Viviane C.H. da Silva[1,2], Viviane Celente[1], Camila Caldana[1,5]

[1]Brazilian Bioethanol Science and Technology Laboratory (CTBE), Brazilian Center for Research in energy and materials (CNPEM)

[2]University of Campinas (UNICAMP)

[3]University of Viçosa (UFV)

[4]CTBE, CNPEM

[5]Brazilian Bioethanol Science and Technology Laboratory (CTBE/CNPEM), Max Planck Partner Group

*These authors contributed equally

Correspondence to: Camila Caldana at caldana@mpimp-golm.mpg.de

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Abstract

A wide range of studies in plant biology are performed using hydroponic cultures. In this work, an *in vitro* hydroponic growth system designed for assessing plant responses to chemicals and other substances of interest is presented. This system is highly efficient in obtaining homogeneous and healthy seedlings of the C_3 and C_4 model species *Arabidopsis thaliana* and *Setaria viridis*, respectively. The sterile cultivation avoids algae and microorganism contamination, which are known limiting factors for plant normal growth and development in hydroponics. In addition, this system is scalable, enabling the harvest of plant material on a large scale with minor mechanical damage, as well as the harvest of individual parts of a plant if desired. A detailed protocol demonstrating that this system has an easy and low-cost assembly, as it uses pipette racks as the main platform for growing plants, is provided. The feasibility of this system was validated using *Arabidopsis* seedlings to assess the effect of the drug AZD-8055, a chemical inhibitor of the target of rapamycin (TOR) kinase. TOR inhibition was efficiently detected as early as 30 min after an AZD-8055 treatment in roots and shoots. Furthermore, AZD-8055-treated plants displayed the expected starch-excess phenotype. We proposed this hydroponic system as an ideal method for plant researchers aiming to monitor the action of plant inducers or inhibitors, as well as to assess metabolic fluxes using isotope-labeling compounds which, in general, requires the use of expensive reagents.

Video Link

The video component of this article can be found at https://www.jove.com/video/57800/

Introduction

The advantages of growing plants using hydroponics have been widely recognized in the production of large and uniform plants, enabling reproducible experiments[1,2,3]. In this system, the composition of the nutritive solution can be properly controlled and recycled along all stages of plant growth and development. Furthermore, roots are not subjected to abiotic stresses, as can happen in soil-grown plants, such as nutrient starvation and water deficiency[4]. As plants grown hydroponically present morphological and physiological traits fairly similar to the ones cultured in soil, this system has been broadly employed in research because it allows the monitoring of root/shoot growth and their harvesting without injuries[2,5].

Due to the possibility of changing the composition and concentration of the nutritive solution, most of the research using hydroponic conditions has been performed to characterize the functions of micro- and macronutrients[1,3,6,7,8]. However, this system has proved to be very useful to a broad range of applications in plant biology, such as to elucidate the functions of hormones and chemicals in plants. For instance, the discovery of strigolactones as a new class of hormones[9] and the accelerated growth phenotype triggered by brassinosteroid application[10] were performed under hydroponic conditions. Moreover, this system enables experiments with labeled isotopes (*e.g.*, $^{14}N/^{15}N$ and $^{13}CO_2$)[11,12] to evaluate their incorporation into proteins and metabolites by mass spectrometry.

Considering the importance of this system in plant research, a high number of hydroponic cultures has been designed in the last few years, including systems that use (i) the transference of seedlings from plates to hydroponic containers[3,13]; (ii) rockwool that limits access to the early stages of root development[2,14,15]; (iii) polyethylene granulate as the floating body, which makes the homogeneous application of small molecules/treatments difficult[16]; or (iv) a reduced number of plants[9,17]. The volume of hydroponic tanks described in many of those protocols are usually large (small volumes ranging from 1 - 5 L, up to 32 L)[18], which makes the application of chemicals extremely expensive. Although few studies

do describe a hydroponic cultivation under aseptic conditions[8,19], the assembly of the system is usually quite laborious, consisting of the perfect adjustment of nylon meshes into plastic or glass containers[5,8,17,20].

Due to the importance of *Arabidopsis thaliana* as a model plant, the majority of hydroponics systems were designed for this species[1,2,8,14,18,19,20]. Nevertheless, there are a few studies reporting the hydroponic growth features of other plant species with a pretreatment of seeds to improve their germination and synchronization rates *in vitro*[8,16]. In order to work on a large scale, we developed a protocol for setting up a simple and low-cost maintenance hydroponic system that enables sterile conditions for growing plants, including *A. thaliana* and other species, such as the grass *Setaria viridis*. The method described here is suitable for different experiments, as the seedling growth can be maximized, synchronized, and easily monitored. Furthermore, this system has many advantages as: (i) its assembly is straightforward and its components can be reused; (ii) it allows the easy application of different chemicals into the liquid medium; (iii) the seedlings germinate and grow directly in the culture medium without the need of transference to the hydroponics system; (iv) the shoot and root development/growth can be closely supervised and the seedlings are harvested without damages; and (v) it makes it possible to work on a large scale, maintaining physiological conditions.

Protocol

1. Preparation of Liquid and Solid Culture Media

1. Prepare a liquid medium using half-strength Murashige and Skoog (MS) medium with vitamins [0.0125 mg/L of cobalt(II) chloride pentahydrate, 0.0125 mg/L of copper(II) sulfate pentahydrate, 18.35 mg/L of ethylenediaminetetraacetate ferric sodium, 3.10 mg/L of boric acid, 0.415 mg/L of potassium iodide, 8.45 mg/L of manganese sulfate monohydrate, 0.125 mg/L of sodium molybdate dihydrate, 4.30 mg/L of zinc sulfate heptahydrate, 166.01 mg/L of calcium chloride, 85 mg/L of potassium dihydrogen phosphate, 950 mg/L of potassium nitrate, 90.27 mg/L of magnesium sulfate, 825 mg/L of ammonium nitrate, 1 mg/L of glycine, 50 mg/L of myo-inositol, 0.25 mg/L of nicotinic acid, 0.25 mg/L of pyridoxine hydrochloride, and 0.05 mg/L of thiamine hydrochloride] supplemented with 0.25 g/L of MES, and adjust the pH to 5.8 with 10 M KOH.
2. Add 10 g/L of agar to make a half-strength MS-solid medium. Autoclave the medium at 121 °C for 20 min prior to use.

2. Hydroponic System Assembling

NOTE: These steps should be followed meticulously to build the hydroponic system.

1. **Material sterilization**
    1. Pack in the autoclave bag the pipette tip racks (without covers) that will be used as minitanks. Autoclave the racks at 121 °C for 20 min, 15 psi.
    NOTE: The polypropylene pipette tip rack we used had the following dimensions: 120 mm (length) x 89 mm (width) x 55 mm (height). The pipette tip flat surface must have an area for the addition of culture medium. Other tip racks can be used (see **Table of Materials**).
    NOTE: Throughout the assembly procedure of the hydroponic system, it is necessary to use a laminar flow hood, which must be cleaned and disinfected with 70% ethanol prior to use. The experimenter must wear a lab coat, wash their hands and any exposed skin, and disinfect them with 70% ethanol. Gloves are optional, except for drug application.
    2. Clean all the accessories described above (disposable plastic boxes, adhesive tape, pipettes, scissors, and tweezers) with 70% ethanol before entering the laminar flow hood. If the hood allows, turn on the UV light for 10 min prior to the assembling of the hydroponic system in order to keep the work area decontaminated.

2. **Minitank assembling**
    1. Seal the upper surface of the pipette tip flat with adhesive tape (**Figure 1B**). If possible, leave it under UV light for 10 min.
    2. Add 180 µL of melted solid MS culture medium (slightly warm) to each well using a multichannel pipette (**Figure 1C**).
    NOTE: When preparing many tanks, use a hotplate to prevent the MS medium from solidifying.
    3. Allow the medium to solidify completely (for about 30 min).
    NOTE: During the solidification period, the UV light can be turned on.
    4. Fill up the pipette tip rack completely with liquid MS culture medium (**Figure 1D**) and ensure there is close contact between the solid and the liquid media.
    5. Remove the adhesive tapes of the upper surface of the pipette tip flat and fit it on the rack carefully. The hydroponic system is now ready to receive the sterilized seeds.

3. Seed Sterilization

1. Place 500 *Arabidopsis* seeds in a 1.5 mL microtube. Use as many microtubes as necessary according to the number of plants required for the experiment.
2. Wash the seeds with 70% ethanol for 2 min with a gentle agitation. Let the seeds settle down, then remove the ethanol carefully.
3. Add 1 mL of a 10% sodium hypochlorite solution containing 2 µL of a polysorbate 20 detergent. Agitate the solution for 5 min. Remove the solution carefully.
4. Rinse the seeds with sterile distilled water until all the bleach residue is completely removed (approximately 5x).
NOTE: After the surface sterilization, the seeds were immersed in sterile distilled water and stratified at 4 °C in the dark for 5 d to synchronize the germination.
NOTE: Seeds of *Setaria viridis* (accession A10.1) were preincubated in concentrated sulfuric acid for 15 min (to break the physical dormancy), washed thoroughly in sterile distilled water, and then disinfested with a 5% sodium hypochlorite solution containing 0.1% polysorbate 20 for 5 min with a gentle agitation[21]. The remaining sterilization steps were identical to those described for *Arabidopsis* seeds.

4. Seed Application

1. Cut slightly the extremity of a 200 µL tip with the aid of a sterile scalpel.
2. Pipette the *Arabidopsis* seeds into the solid culture medium on the upper surface of the pipette tip flat. Take care that the medium does not loosen from the flat; otherwise, the seeds will be shaded and the seedlings will not grow properly (**Figure 1E**).
   NOTE: Use a sterile tweezer for *Setaria* seeds (with the embryo positioned upward).
3. Store as many minitanks as possible inside a disposable plastic box to maintain a high humidity and keep the environment free from microorganisms (**Figure 1F**).
4. Seal the disposable plastic box thoroughly using adhesive tape to avoid contamination.
5. Place the hydroponic systems into a growth chamber with the appropriate growth conditions for the plant of interest.
   NOTE: In this work, the following conditions were used: 75% of humidity, and 150 µmol m^{-2} s^{-1} of irradiance and equinoctial conditions of 12 h light (21 °C)/12 h dark (19 °C) for *Arabidopsis*, or 300 µmol m^{-2} s^{-1} of irradiance and 12 h light (28 °C)/12 h dark (25 °C) for *Setaria* (**Figure 1G** and **1H**).

5. Validating the Use of this Hydroponic System to Inhibit the Target of Rapamycin Kinase

Note: This hydroponic system was initially developed to facilitate the administration of chemicals to plants, which, in general, are very expensive to be applied in large-scale experiments. As a proof of concept, the ATP-competitive inhibitor AZD-8055, which is known to specifically target the ATP binding site of the TOR protein kinase[22], was employed to follow the repression of TOR activity in seedlings of *A. thaliana* Columbia-0 (The Nottingham Arabidopsis Stock Centre, NASC ID: N22681). Here, the protocol used is briefly described.

1. Grow seeds hydroponically until stage 1.04 according to the BBCH scale[23] (for about 11 d) under the climatic conditions described above. Replace the nutrient solution, either with fresh medium containing 0.05% DMSO (control), 2 µM AZD-8055 (TOR inhibitor) diluted in DMSO, or without treatment (mock), at the end of the night (EN).
2. Harvest some seedlings at different time points after the treatment and separate them into roots and shoots. Freeze the samples in liquid nitrogen, grind them to a fine powder in a robotic grinder (see **Table of Materials**), and store the powder at -80 °C until use.
3. Immunoblot against phosphorylated and non-phosphorylated forms of 40S ribosomal protein S6 (RPS6) according to Dobrenel *et al.*[24].
4. Bleach intact seedlings for sample depigmentation, wash them in distilled water, immerse them in an iodine solution for 5 min[25], and photograph the seedlings in a stereomicroscope (0.63X objective, 20x approximation, and 7.5x magnitude) for a qualitative assessment of the starch content.
5. Quantify the starch following the enzymatic degradation and measurement of the released glucose spectrophotometrically by coupling it to the reduction of $NADP^+$ to NADPH[26,27].
6. Perform a total RNA extraction, a cDNA synthesis, and quantitative RT-PCR assays as described by Caldana *et al.*[28] to evaluate the expression level of genes related to different sorts of stresses.
7. Optionally, grow seedlings on a horticultural substrate in plastic pots with a 0.1 L capacity under similar climatic conditions [60 % of humidity, 150 µmol m^{-2} s^{-1} of irradiance, and equinoctial conditions of 12 h light (21 °C)/12 h dark (19 °C)] in order to compare them with seedlings grown hydroponically.
   NOTE: The target genes used for the gene expression assays were *ABF3* (At4g34000), *ASN1* (At3g47340), and *TPS5* (At4g17770), and their expression levels were normalized employing the delta-Ct method[29] using *ACT2* (At3g18780) or *PDF2* (At4g04890) as the internal reference genes, assuming 100% of PCR amplification efficiency across all samples. The oligonucleotide pairs used for the quantitative PCR were: *ABF3* (GTTCTCAACCTGCAACACAGTGC; TCCAGGAGATACTGCTGCAACC), *ASN1* (AGGTGCGGACGAGATCTTTG; GTGAAGAGCCTTGATCTTGC), *TPS5* (CTGCTCTGATGCTCCTTCTTCC; AAGCTGGTTTCCAACGATGATG), *ACT2* (CGTACAACCGGTATTGTGCTGG; CTCTCTCTGTAAGGATCTTCATG), and *PDF2* (TAACGTGGCCAAAATGATGC; GTTCTCCACAACCGCTTGGT).

Representative Results

The TOR kinase is a major regulator that integrates nutrient and energy signaling to promote cell proliferation and growth in all eukaryotes. Efforts to elucidate TOR functions in plants include the generation of *Arabidopsis* transgenic lines containing TOR conditional repression through RNA interference or artificial microRNA[28,30,31], given the embryo lethal phenotype of TOR knockout plants[32,33,34,35]. Most of the conditional transgenic lines are under the control of estradiol-, dexamethasone-, or ethanol-inducible promoters, which could also make use of this hydroponic system.

One of the well-known targets of TOR activity in *Arabidopsis* is the direct phosphorylation of the ribosomal protein S6 kinase (S6K)[34,36,37,38]. Upon phosphorylation, S6K further phosphorylates the 40S ribosomal protein S6 (RPS6), affecting the ribosomal protein translation[24,39,40]. Recently, it has been demonstrated that the phosphorylation of an RPS6 Ser240 site is a good marker of TOR activity[24]. Immunoblotting assays confirmed that soon after 30 min of drug administration, a significant decrease in the Ser240 phosphorylation was observed in both roots and shoots (**Figure 2**). Under the experimental conditions used, AZD-8055 has also shown to be a potent TOR inhibitor, which rapidly represses its kinase activity.

Transgenic *Arabidopsis* lines with a reduced expression of the TOR gene or components of the TOR complex present a clear starch excess phenotype[28,31]. Qualitative analysis of starch using Lugol's solution revealed the expected pattern of starch accumulation and degradation during the diel cycle (**Figure 3**). Seedlings that did not receive an application of DMSO or AZD-8055 showed no greater accumulation of starch in their leaves at the end of the night (EN), and the starch accumulation in the control plants (which received 0.05% DMSO) was consistent with the literature[41,42]. Furthermore, plants treated with AZD-8055 presented a greater amount of remaining starch at the EN when compared to the control seedlings. These results indicated the usefulness of the proposed hydroponic system in growing seedlings mimicking physiological conditions. This system also enabled the confirmation of the starch excess phenotype typical of a repression of the TOR complex components[24,28,31].

Starch content was also accurately measured using a sensitive methodology, demonstrating that the AZD-8055 treatment led to seedlings containing significantly higher levels of starch at both the end of the day (ED) and EN in comparison to the DMSO-treated control plants (**Figure 4**). Starch accumulates in the leaves during the day and is remobilized overnight to sustain metabolic activity, mainly the respiration and the continuous export of sucrose to other plant organs[41,42]. Under normal conditions, only a small fraction of starch (between 5% and 10% of the amount at the ED) remains at the EN[43,44,45]. These results attested that the starch excess phenotype observed under the TOR repression occurs all over the diel cycle.

Hydroponically grown plants were compared to seedlings grown in a horticultural substrate under very similar climatic conditions concerning the expression level of the abscisic acid-responsive element-binding factor 3 (*ABF3*) gene (**Figure 5A**), which directly correlates with internal ABA levels, a class of hormones widely known as a marker due to its role in multiple abiotic stress responses[46,47,48]. Although seedlings grown in the hydroponic system did present a significant increase in the level of *ABF3*, the expression of asparagine synthase 1 (*ASN1*) was not affected by the DMSO or AZD treatments (**Figure 5B**). However, trehalose phosphate synthase 5 (*TPS5*) was significantly increased after 8 h of TOR inhibition (**Figure 5B**). *ASN1* and *TPS5* respond to low and high sugar levels[49,50,51,52,53,54], respectively, suggesting that these plants were not experiencing energetic stress.

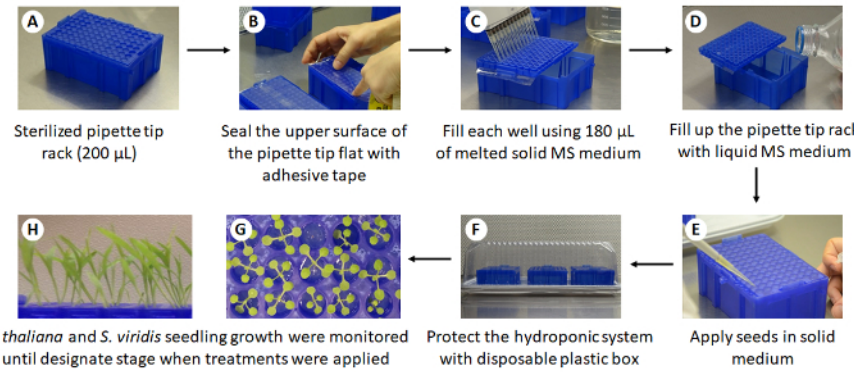


Figure 1: Workflow for assembling the hydroponic system. Please click here to view a larger version of this figure.

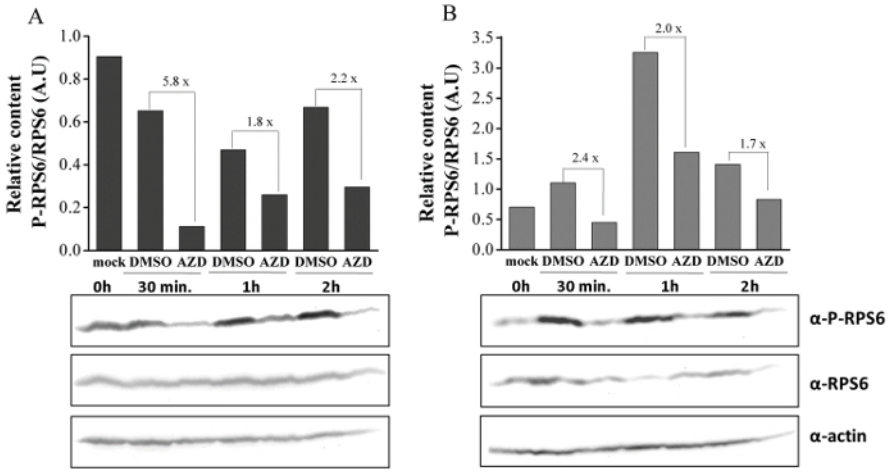


Figure 2: Effect of TOR inhibition on the RPS6 phosphorylation in different tissues of *Arabidopsis thaliana*. Immunoblotting shows the abundance of the total and phosphorylated RPS6 in the (**A**) root and (**B**) shoot extracts of seedlings treated with 2 µM AZD-8055 or 0.05% DMSO (control). Values represent the ratios normalized by the non-phosphorylated protein RPS6. Anti-actin antibody was used as a loading control. Please click here to view a larger version of this figure.

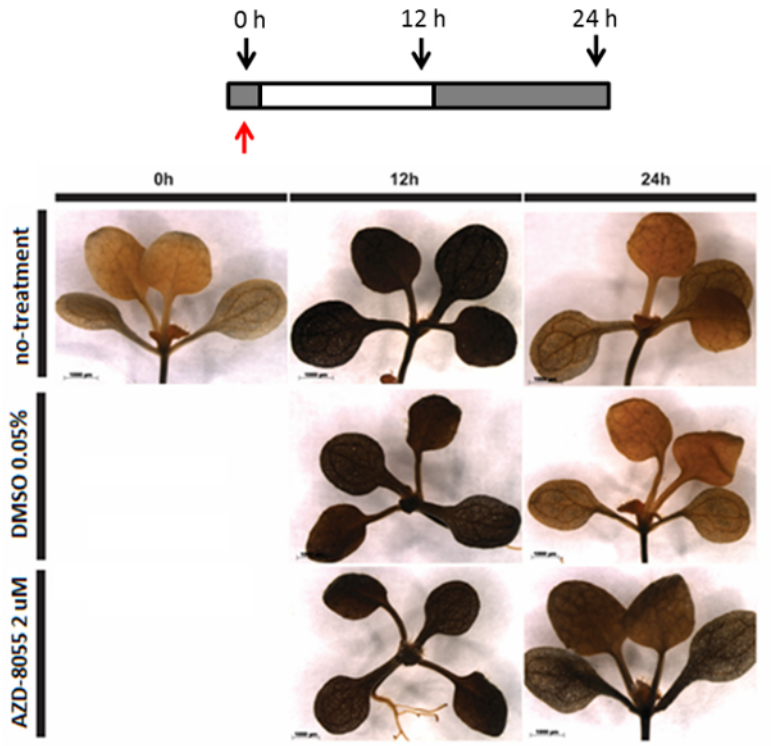


Figure 3: *Arabidopsis thaliana* seedlings stained with Lugol's reagent. Treatments with 2 µM AZD-8055 or 0.05% DMSO (control) were applied at the EN (red arrow) and compared to mock seedlings (no-treatment). Seedlings were harvested before the treatment application (0 h) and at 12 h (ED) and 24 h (EN) after the treatment, indicated by black arrows. Please click here to view a larger version of this figure.

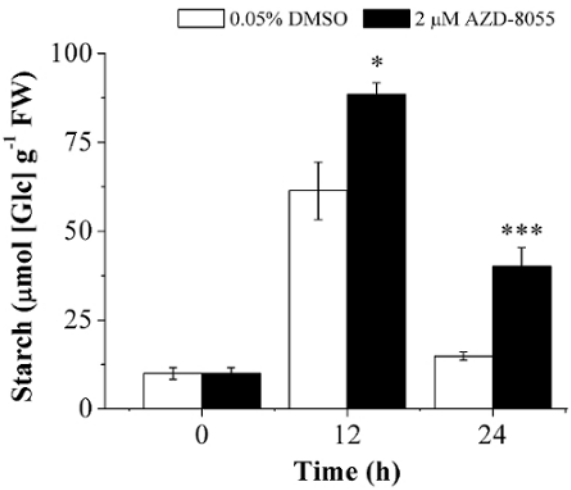


Figure 4: Effect of TOR inhibition on the starch content of *Arabidopsis thaliana* seedlings. Starch was measured enzymatically before (0 h) and at 12 h (ED) or 24 h (EN) after the treatment with 2 µM AZD-8055 (black) or 0.05% DMSO (control, white). The values shown are the mean ± the standard error (SE) (n = 4). Significant differences between seedlings treated with AZD-8055 and DMSO, using Student's t-test, are indicated by asterisks: * (P < 0.05) and *** (P < 0.001). Please click here to view a larger version of this figure.

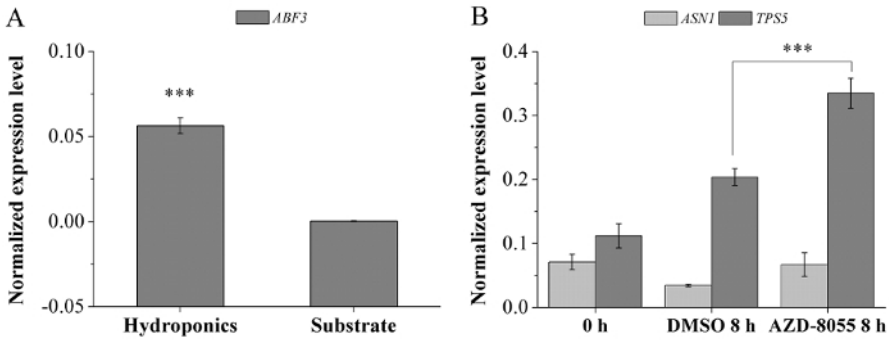
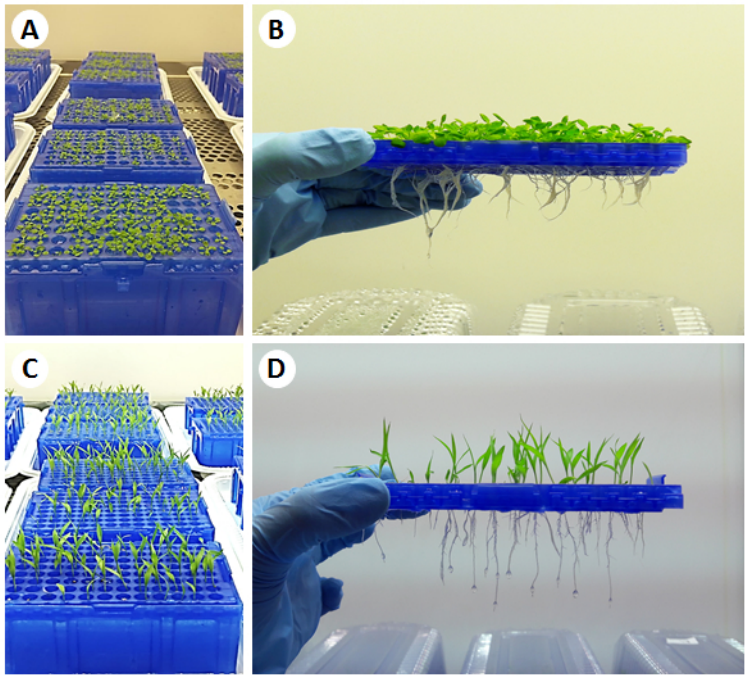


Figure 5: Expression level of stress-related genes. (**A**) Comparison of *ABF3* transcripts in hydroponically and substrate-grown *Arabidopsis* Col-0 seedlings. (**B**) Comparison of *ASN1* and *TPS5* transcripts in *Arabidopsis* seedlings treated with 2 µM AZD-8055 and 0.05% DMSO. The normalized expression levels are shown as 2^(-dCt). The values shown are the mean ± SE (*n* = 3). Significant differences, using Student's *t*-test, are indicated by asterisks: *** ($P < 0.001$). Please click here to view a larger version of this figure.



Supplementary Figure 1:This *in vitro* hydroponic system makes it possible to synchronize germination and obtain homogeneous seedlings. Seeds of *A. thaliana* (C3) and *S. viridis* (C4) were germinated directly in this system. (**A** and **C**) The seedlings were homogeneous in relation to the developmental stage and the treatment was applied after 11 d (*Arabidopsis*) or 7 d (*Setaria*). (**B** and **D**) The roots grow directly toward the nutritive solution, facilitating the addition of different substances and their absorption. These results strongly indicate that this system offers an optimal environment for plant growth and can be used to efficiently perform a wide range of assays. In addition, this hydroponic system is very useful for large-scale experiments.

Discussion

This optimized hydroponic structure enables the successful *in vitro* culture of plants. Seeds germinate well on the solid medium at the pipette tip flat surface, a considerable gain in comparison to systems where seeds are soaked with the nutrient solution. A great advantage of this system is that during the seedling development, roots get directly in contact with the liquid medium without the need of transference. Moreover, chemical treatment can be easily applied in the liquid medium in a reduced volume. Humidity is kept high, avoiding the evaporation of the nutrient solution and its replenishment. In addition, homogenous growth and development during the seedling establishment can be easily obtained, and aeration is not required when working with small tanks and seedlings at this developmental stage[1,10,18]. In order to guarantee that the system will be completely free from contaminants, a critical step is the sterilization of any material used and intensive care during its assembly. Due to the impossibility to sterilize some components in the autoclave (*e.g.*, disposable plastic boxes), it is strongly recommended to first clear them with 70% ethanol and then apply a short period of UV light before use. In our experience, the use of UV light, after sealing the flat surface with adhesive tape and during the media solidification, also avoids bacterial and fungal contamination. Furthermore, be cautious not to touch the media, always moving the pipette racks by its lateral side.

To assure the optimal growth of the seedlings, it is important to monitor the close contact between solid and liquid media, ensuring the complete immersion of the roots after the seed germination. The solid medium must be adequately dense (10 g/L agar) and totally solidified so as not to loosen from the flat surface and float into the nutrient solution. Besides *Arabidopsis*, this system can be used for growing other plant species,

as long as the seeds are small enough to fit the wells of the flat. In this sense, the hydroponic method presented here was also efficient for growing *Setaria viridis*, a small grass that has recently emerged as a novel model system for studying C_4 photosynthesis, stress biology, and other bioenergy crop traits[55]. Similar to *Arabidopsis*, this system allows to produce uniformly growing *Setaria* seedlings with a good root system and on a large scale (**Supplementary Figure 1)**, because every rack supports 96 seeds, ensuring many seedlings per biological replicate and, consequently, sufficient material for a myriad of downstream applications. A higher number of replicates increases the efficiency of statistical testing, leading to more accurate and reliable results in experimental studies[56]. For example, using a growth chamber with an area of only 1.5 m^2, we were able to grow 6,000 seedlings simultaneously, making it possible to perform temporal kinetics of the response to a desired treatment. Additionally, the harvested samples can be used for multiple and complementary 'omics' analyses that can demand a great amount of tissue (*e.g.*, immunoblotting). This hydroponic structure is of special interest for groups aiming to analyze distinct plant organs (*e.g.*, roots and shoots), because it enables their easy and fast separation.

A small number of studies described the use of pipette tip boxes during the initial plant development prior to a transference to bigger hydroponic tanks[11,20], and more recently, a very similar system was employed to evaluate the amino acid uptake and translocation in 5-week-old *Arabidopsis* plants[57]. The protocol described here provides additional benefits in terms of cultivating the plants under sterile conditions.

Although this system was initially developed to grow seedlings, it could also be suitable for bigger plants. In this scenario, it is worth mentioning that care must be taken to place the seeds more distant from each other to avoid as much shading as possible during growth. Furthermore, aeration can be introduced into the racks to prevent hypoxia through one well of the pipette tip flat, a common problem in submerged *Arabidopsis* roots growing for longer periods. Due to their sessile nature, plants are subjected to several sorts of abiotic and biotic stresses, depending on their surrounding environment. Therefore, considering the aim of the study and the developmental stage, it might be important to monitor if the plants growing in this system are suffering from some sort of stress.

The results presented here have shown that this hydroponic system is very useful for the application of chemicals to the nutritive solution, particularly when working with expensive substances, due to the small volume of the pipette tip racks. We have succeeded in using this system to effectively repress the activity of TOR kinase by AZD-8055 and confirmed that the phosphorylation status of its downstream target RPS6 is already affected after 30 min of treatment application. Moreover, TOR inhibition leads to seedlings containing higher starch levels during the day and night in comparison to the control seedlings. Such assays can be easily employed to extend the observations already obtained with transgenic lines, allowing an inducible repression of the gene-encoding components of the TOR complex, or any other pathway of interest. In summary, the proposed hydroponic system possesses many advantages because it is very easy and simple to assemble, has a low cost (the major components are cheap and can be extensively reused), is versatile (enables the study of intact seedlings or distinct tissues, in specific or along plant developmental stages), and is highly scalable (it allows the cultivation of a huge number of seedlings in a very small area).

Disclosures

The authors have nothing to disclose.

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