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Establishment of a rapid split-root assay in hydroponic conditions for eight upland cotton varieties

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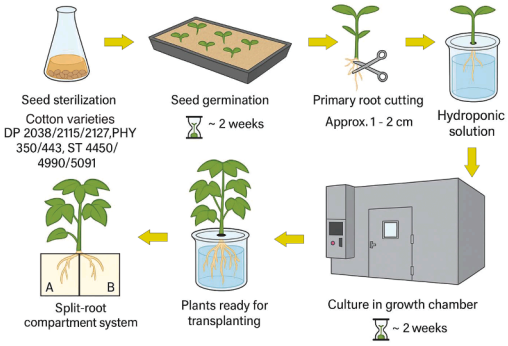
GRAPHICAL ABSTRACT



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ABSTRACT

Upland cotton (*Gossypium hirsutum* L.) is a major crop in the United States. Understanding how cotton roots develop and respond to abiotic and biotic factors is crucial for improving nutrient acquisition, enhancing crop resilience under stress, and optimizing overall crop production. Split-root techniques have been developed for numerous plant species, providing a controlled framework for monitoring root development, and investigating systemic and local plant responses to various environmental factors. However, a standardized cotton-specific protocol optimized for laboratory studies has yet to be established. This protocol facilitates the rapid establishment of split-root systems in eight upland cotton varieties within four weeks after germination. This is accomplished by cutting the primary root and immediately transplanting the seedlings into hydroponic conditions to promote lateral root growth, after which the root system can be divided

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equally into separate compartments. Once established, each compartment can be subjected to different, independent treatments. This method was validated across all eight varieties by quantifying the difference in root dry weight between the two halves of each plant's root system and analyzing those differences across varieties. Statistical analysis was performed and Kruskal-Wallis and Wilcoxon signed-rant tests confirmed no significant difference between the roots of the two sides for any cultivar, thus confirming this method's reliability.

- We developed a standardized split-root protocol tailored for upland cotton using hydroponics.
- This protocol was performed on eight varieties within four weeks after germination.
- We validated the method by comparing root biomass distribution between compartments to confirm reliability.

Specifications table

Subject area	Agricultural and Biological Sciences
More specific subject area	*Root physiology*
Name of your method	*Split-root assays for cotton plants*
Name and reference of original method	*N/A*
Resource availability	*Reagents and Equipment are listed in the Materials section*

Background

Upland cotton (*Gossypium hirsutum* L.) production is pivotal to the United States economy. As of today, Texas (30 %), Georgia (16 %), Arkansas (9 %), Mississippi (7 %), and North Carolina (6 %) are the top five cotton-producing states [1]. In the last five years, harvested area varied from 2.6 to 4.7 million hectares and average lint yield ranged from 900 to 1070 kg/ha. Such production resulted in the US ranking as the 4th top global producer, responsible for 10.6 % of all cotton produced in the world, and the 2nd largest exporter, accounting for 28 % of world cotton exports as of 2025 [1]. This amounted to a global export market of $5.7 billions of dollars in 2021, and $8.9 billions of dollars in 2022 [2]. Understanding the development of its roots and their interactions with the soil is crucial for improving nutrient uptake, enhancing stress tolerance, and optimizing overall plant productivity.

Plant roots are dynamic and physiologically active organs that interact with various environments and are exposed to diverse and changing soil conditions. To investigate root responses to biotic and abiotic factors and understand the communication within the soil-root-shoot continuum, split-root techniques have been developed in multiple plant species [3–4]. This approach allows the separation of a single plant's root system into two distinct portions, each grown under different conditions, thereby enabling the differentiation between local and systemic responses. The use of such systems contributes significantly to the understanding of root metabolism and signaling processes in a range of plant species [3–7].

A few publications reported the use of split-root systems in cotton, either using grafting [8] or directly in pots filled with sand [9–10], but no standardized method for laboratory-oriented use involving multiple varieties has been described so far. These existing approaches are often limited by reduced control over the root environment that could lead to contaminations, greater difficulty in harvesting clean roots from sandy substrates, and higher space and material requirements, which can hinder reproducibility and large-scale testing. Here, we report a rapid split-root method tested with eight cotton varieties that involves the use of a simplified hydroponic system to promote lateral root elongation. This process was completed in under four weeks post germination. Our method provides a versatile technique for investigating a range of experimental conditions, including nutrient dynamics, drought stress, plant-microbe interactions, and plant-insect interactions, contributing to a deeper understanding of root metabolic responses in cotton.

Materials

(1). Forceps, Fisherbrand™ filter/membrane forceps or similar (Fisher Scientific #09–73–50)
(2). Scissors, Fisherbrand™ utility scissors or similar (Fisher Scientific #08–945)
(3). 250 mL glass beakers (Kimax, USA, No. 14,000)
(4). Clone collars (Growneer®, diameter 6.985 cm, thickness 1.905 cm, 8 spokes).
(5). 1000 µL pipette tips with filters (USA Scientific, 1122–1830), sterile
(6). Aluminum foil
(7). Paper tape
(8). Plastic planting tubs (25.4 cm x 50.8 cm x 5.5 cm; L x W x H, Rubbermaid Pan, 11.4-Quart, White)
(9). Plastic domes (Yield Lab propagation and humidity vented domes, 17.8 cm height, to fit planting trays)
(10). SafeT Sorb substrate (Ep Minerals® #7941) for potting medium
(11). Sand, Lowes Home Improvement, (QUIKRETE 0.5-cu ft 50-lb Sand #98,545)

(12). Perlite, (Sunleaves, Horticultural coarse perlite, PVP Industries Organic Perlite Planting Soil Additive, White)
(13). Vermiculite, (Viagrow Horticultural vermiculite, #5970,487)
(14). Plastic coffee stir rods (KBG, 3-hole disposable coffee straw, 17 cm)
(15). Cable ties (TANTII® Industrial zip ties, 20.32 cm) sterilized with 90 % ethanol
(16). Plastic planting drip trays (25.4 cm x 50.8 cm x 5.5 cm; L x W x H)
(17). Plastic planting boxes (12 cm × 8 cm x 8 cm; L x W x H; from Carmo A/S, Series 500/80: https://ultraplast.dk/wp-content/uploads/2023/06/Ultraplast_Series-500–80.pdf). Six 5 mm holes were drilled in the bottom of each pot.
(18). Spatula, Eisco Stainless Steel Laboratory Spatula (Fisher Scientific #S41699)

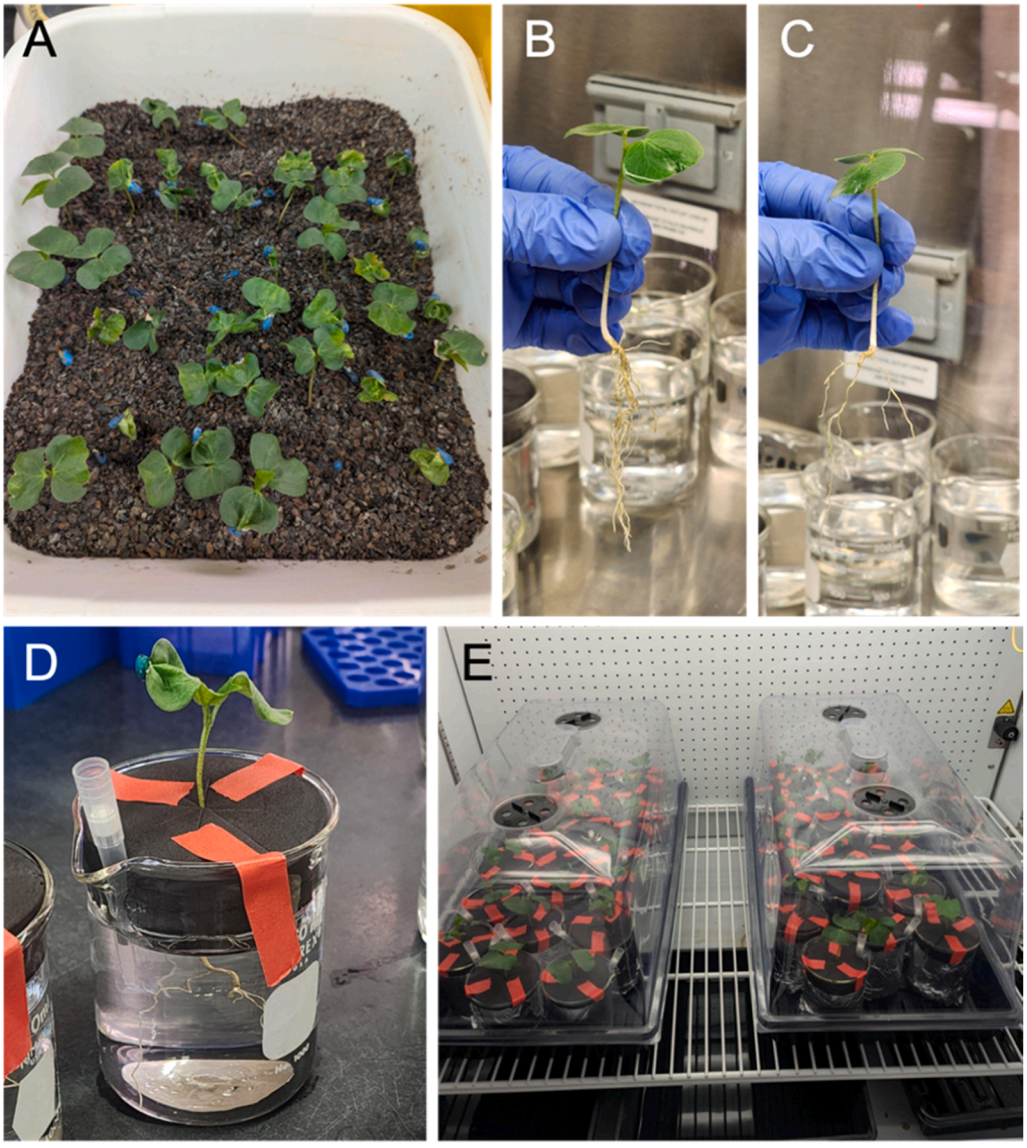

Fig. 1. Seed germination and preparation of hydroponic systems for cotton varieties. **A.** Sterilized seeds of 8 cotton varieties were germinated in tubs containing moist Safe T Sorb and left under grow lights for approximately 2 weeks. **B.** A germinated seedling was removed from the substrate and cleaned with milli-Q water. **C.** The lateral roots were separated from the primary root, and the primary root was cut to promote lateral growth. **D.** The seedling was transferred to a sterile clone collar and placed in a 250 mL beaker filled with Long Ashton solution. Then, the clone collar was secured with paper tape and a sterile 1000 μL filtered pipette tip was inserted between the collar and beaker to promote gas exchange. **E.** The hydroponic systems were transferred to a growth chamber (16 h of light day, 8 h of dark, 25–27 °C, light intensity of 210 μmol m^{-2} s^{-1}) until lateral roots reached the bottom of the beakers.

Method details

Cotton seeds of eight varieties were used: Stoneville 4990, Stoneville 4550, Stoneville 5091, Deltapine 2038, Phytogen 433, DeltaPine 2115, Phytogen 360, DeltaPine 2127. They were stored at 4 °C before they were germinated. Fifty seeds per cultivar were sterilized for germination. Since the following protocol has been designed for laboratory-oriented studies in controlled environments, it is important to minimize microbial contamination. Therefore, we followed aseptic handling throughout all stages of the experiment. Most tools, the hydroponic solution, and the substrate used in the split-root systems were autoclaved. Clone collars were sterilized with ethanol and UV light, and seedlings were transferred from the biosafety cabinet to the growth chamber under a clear plastic dome to limit microbial exposure. Details are mentioned below.

Seed sterilization

1. In a biosafety cabinet, use an autoclaved 250 mL beaker, and add 200 mL 0.6 % bleach (sodium hypochlorite) solution. Add seeds to sterilize for 4 min.
2. Drain bleach solution and rinse seeds 3 times with milli-Q water.
3. After the 3rd rinse, let the seeds sit in milli-Q water for 4 min.
4. Drain water and lay the seeds between dry and sterile paper towels for them to dry. Cover it on the top with paper towel to avoid contamination.
5. Once dried, store them in tubes inside the refrigerator (4 °C) until use.

Seed germination

Seed germination can be done using various methods. The method described here was used for this protocol. If using a growth chamber, set your growth chamber settings at least a day before to allow time for the chamber to adjust. Seeds will germinate in about 5–10 days, and they will show true leaves about 5–10 days after germination. Seedling transplant should happen around day 14 or when the first true leaves emerge.

1. Wash the substrate with deionized water until it runs clear, making sure to drain excess water.
2. Sanitize germination tubs using a 10 % bleach solution. Let the tubs soak for 10 min. Rinse 5 times, ensuring no bleach residue. Germination trays should be deep enough to allow the primary root to grow down freely.
3. Fill a tub with a moist substrate to a depth of 10 cm.
4. Make small holes on the surface with a spatula to sow seeds.
5. Plant seeds radicle down, 1–2 cm in the soil, 1 seed per hole.
6. Place the tubs under grow lights at room temperature (20–25 °C) for approximately 2 weeks or until the seedlings begin to grow true leaves as shown in Fig. 1A.
7. Use 400 mL milli-Q water per tub every 2 days to keep it moist. Adjust accordingly if the substrate is too damp or too dry. The soil should be kept moist but not soaking.

Preparation of hydroponic material and nutrient solution

Before transplanting, sterilize your equipment. Autoclave forceps, scissors, pipette tips, glass beakers, and an autoclavable container to hold your sterilized clone collars. One glass beaker, one clone collar, and one 1000 µL pipette tip with filter will be used per seedling.

1. In a biosafety cabinet, extend the opening of each clone collar slightly and submerge in a 90 % ethanol solution for 10 min. Sterile tubs can be used to submerge many at a time.
2. Remove collars from ethanol and set them upright with their openings facing upward. Allow them to sit upright under UV for 1 hour. After 1 hour, lay them flat under the UV for another hour. Flip them to expose the other side to UV for an additional hour.
3. Check that the ethanol is completely evaporated from the rubber before storing them in your sterilized container.

Prepare the nutrient solution for the hydroponic system. A modified Long Ashton solution was used for this protocol (Table S1).

Transplantation into a hydroponic system and maintenance

1. Under a biosafety cabinet, add 200 mL of the nutrient solution to each autoclaved 250 mL beaker.
2. Gently pull up a healthy seedling by the base of the stem, clean the roots with milli-Q water to remove the substrate (see Fig. 1B).
3. Wearing sterile gloves, find and cut the primary root of each seedling with sterile scissors to promote lateral root growth (approximately 1–2 cm) (Fig. 1C). You can take a slightly aggressive approach, cutting off up to 50 % of roots. This step is crucial to inhibit the dominance of the primary root and promote lateral root branching and elongation.
4. Place the plant in a sterilized clone collar, ensuring that the top and bottom of the clone collar have shoot and root tissues extending respectively from it.

5. With the seedling in place, put the clone collar on top of the beaker and press it in evenly until the bottom of the clone collar is wet from the top of the nutrient solution. Make sure that the roots are fully immersed in the nutrient solution,
6. Insert one sterile 1000 μL pipette tip with a filter, positioning it against the inner wall of the beaker so that half of the tip remains outside the beaker and half inside as shown in Fig. 1D To broaden its opening, make a cut with sterile scissors about 0.75 cm from the tip. This setup ensures sufficient gas exchange between the inside and outside of the beaker to prevent root anoxia while maintaining a semi-contained environment.
7. Secure the clone collar in the beaker with small pieces of paper tape on 3 sides of the outer edge.
8. Wrap the beaker with aluminum foil around the outer surface of the beaker to prevent light exposure to the roots.
9. Repeat the process for all the seedlings.
10. Carefully place all the transplanted beakers in sterilized and sturdy plastic planting trays (25.4 cm x 50.8 cm x 5.5 cm; L x W x H).
11. Cover the trays with plastic domes to prevent transplantation shock to the seedlings in the growth chamber. Open the dome ventilation halfway.
12. Place the prepared trays in the growth chamber (see Fig. 1E) set to 16-hour day and 8-hour night cycle, 25–27 °C temperature, light intensity to 210 μmol m^{-2} s^{-1}, and relative humidity maintained to approximately 65 %−70 % by placing several trays of sterile milli-Q water and covering the seedlings with a clear humidity dome.
13. Keep the seedlings in hydroponic beaker systems in the growth chamber for a total of 7–14 days. The roots should be around 10–15 cm in length or reaching the bottom of the beaker for transplant (see Fig. 2A). If growth is slower than expected, extend hydroponic culture until one of the two above criteria is met. Refresh the hydroponic solution every two weeks to maintain solution quality and nutrient availability. If growth is faster, transplant earlier when one of the two criteria is met.
14. Every 36 h, lightly shake the trays for 1 min to encourage oxygen diffusion into the nutrient solution and prevent anoxia.

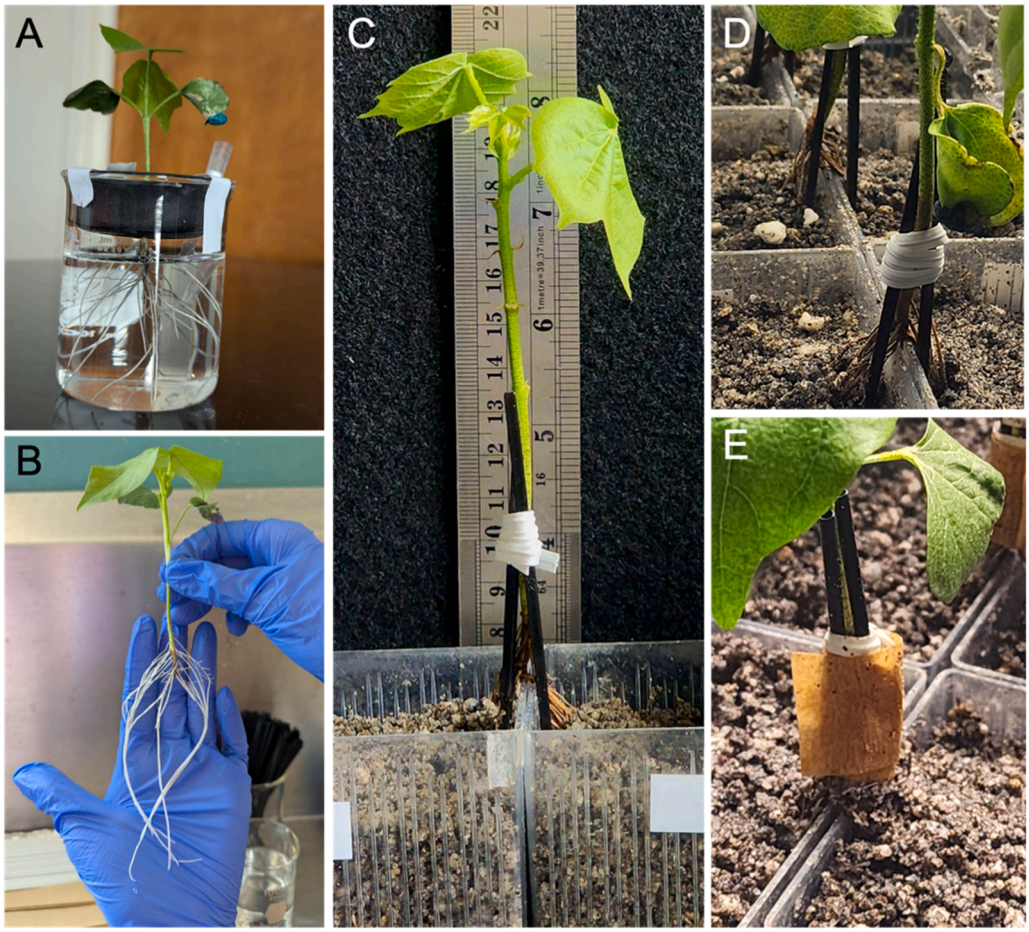

Fig. 2. Hydroponics system into split-root compartments. A. A cotton seedling ready for transplant into the two-compartment boxes, with roots reaching the bottom of the 250 mL beaker (approximately 10–15 cm). **B.** The lateral root system of the seedling was divided into two equivalent sections, teasing apart the lateral roots and aiming for a visually similar volume of root mass on each side. **C and D.** Two coffee stirrers, one in each compartment, were placed into the soil to keep the plant upright. A tie was wrapped around the stem to stabilize everything in place. **E.** Wet germination paper was wrapped around the exposed roots to help prevent transplant shock.

15. Remove the plastic domes at day 7.

Preparation of transplantation into split-root compartments

Prepare compartments

1. Sterilize compartments and planting trays a day before transplant. Submerge them in a 1:10 bleach dilution for 8 minutes. Rinse each item thoroughly 3 times to remove any residual bleach. Place compartments upside down to dry overnight.
2. On the day of transplant, ensure the compartments have dried completely so that no residual bleach remains.

Transplant seedlings into split-root compartments

Ensure that the lateral root system of the seedlings has reached the bottom of the 250 mL beaker before transplanting into split-root compartments. Time in the hydroponic system may be lengthened to allow for more root growth if necessary. To maintained root moisture during transplantation, it is important to work on one plant at a time while the rest stayed under the plastic dome. In the case of unexpected delay, briefly place the roots in sterile deionized water. This is especially important during transplantation of seedlings from hydroponic solution to split-root soil systems. Avoid root desiccation, which is visibly shown through tissue shrinkage and increased brittleness.

1. Arrange two appropriately labeled compartment units side-by-side. For the purposes of this protocol, the left compartment unit will be referred to as root compartment A, and the right as root compartment B. Place all compartments into drip trays prior to transplant to prevent root disturbance.
2. Wearing sterile gloves, place approximately 600 cm^3 of moist soil into each compartment. Adjust the soil level so that the top of the soil is within 2 cm from the top of the compartment.
3. Create a hole with a sterile spatula in root compartment A and root compartment B, approximately 2 cm wide and 5 cm deep. The holes should be about 1 cm away from the center of the adjoining walls.
4. Working one plant at a time to ensure the roots remain hydrated, remove a plant from the hydroponic system. Gently tease apart the lateral roots, aiming for a visually similar volume of root mass on each side as shown in Fig. 2B.

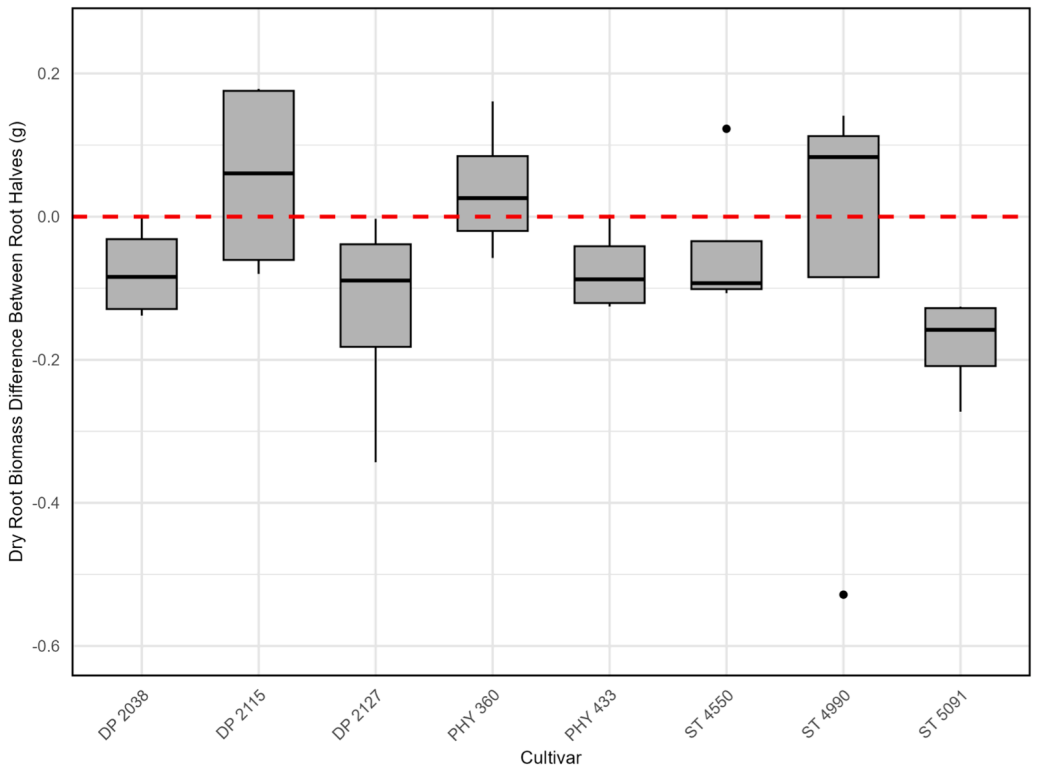

Fig. 3. Dry root biomass comparison between root halves of each plant across eight varieties of cotton *(Gossypium hirsutum L)*. Dry weight was recorded for each half of the plant. The difference between both sides of the root system was calculated for every plant, with four plants per cultivar. A Kruskal-Wallis test showed no significant difference between each root half across varieties.

5. Gently place one half of the root system into root compartment A. Once the roots are placed in the hole, cover the roots with soil using the spatula.
6. Place the other half of the roots into the hole of root compartment B and fill with soil.
7. Hold the plant upright, in the center between the two compartments. Use coffee stirrers, one in each compartment, to stabilize the plant. Wrap a tie around the stem and both coffee stirrers to keep everything stable (see Fig. 2C-D).
8. Wet germination paper and wrap it around the exposed roots to keep them moist (Fig. 2E).
9. Once finished, cover the trays with plastic domes and place them back into the growth chamber set to 16-hour day and 8-hour night cycle, 25-27 °C temperature, light intensity to 210 μmol m^{-2} s^{-1}, and relative humidity maintained to approximately 65 %-70 %. Keep the substrate moist and monitor closely. Remove domes seven days after transplant.

Method validation

The split-root protocol for cotton was validated by harvesting the cotton plants eight weeks post-transplantation and confirming that root growth was comparable between both sides of the root system of each plant. The roots were cut just below the stem at the point where they split into two roots. Each of the root halves was processed individually to avoid any cross-contamination. Roots were carefully removed from the compartments by gently inverting the substrate while holding the root to avoid the risk of breaking or losing roots. Roots were then cleaned by immersing them in deionized water to remove all the substrate from them. Excess moisture from the roots was absorbed using paper towels. Roots were placed into an oven in 50 mL tubes with caps off for 5 days at 65 °C for dry weight measurement (Fig. 3). For statistical analysis, a Kruskal-Wallis test, a non-parametric alternative to ANOVA, was used as the data did not meet the assumption of normality based on Shapiro-Wilk tests (Fig. 3). Pairwise comparisons between compartments were further evaluated using the Wilcoxon signed-rank test (Table S2). There was no significant difference in the biomass between the two root halves of each plant's root system across all eight of the varieties. This reliability ensures that any observed effects from differential treatments can be attributed to the treatments themselves, not asymmetric root growth. Hence, we report a standardized split-root protocol for cotton that allows controlled investigation of systemic vs. local signaling in response to diverse treatments such as abiotic stressors, beneficial microbes interactions, pathogen interactions, or differential nutrient applications.

Discussion

Although studies on a variety of plant species have utilized variations of the split-root method, no standardized protocol for split-root assays designed for cotton has been developed so far. One such study was done to investigate the systematic capabilities of *Bacillus* to control the population of nematodes in the cotton cultivar Phytogen 333 WRF [10]. In their split-root approach, they cut the root tip 6 days after seeds germinated or immediately after a small tap root was formed. Then, the seedlings were grown in a pot at the rate of 4 seedlings per plot in the greenhouse for 1–2 weeks until they had equivalent root halves to proceed with the establishment of a split-root setup in two planting boxes. In contrast, our split-root protocol, which was tested using eight varieties of cotton, uses hydroponic solutions. We cut around 50 % of the entire root system, or 1–2 cm of the main root, to promote lateral root growth in a hydroponic system using Long Ashton as the nutrient solution. This hydroponic phase accelerates lateral root development, enabling the establishment of a split-root system in roughly 4 weeks—faster than the traditional method, which requires greenhouse growth in addition to 1–2 weeks of preparation. Moreover, maintaining seedlings in a controlled laboratory environment reduces variability from environmental factors and enhances reproducibility compared with greenhouse-based setups. A similar approach was also recommended by Saiz-Fernández et al. [7] for smaller plants like *Arabidopsis thaliana*, where they described partial de-rooting as a technique that allows less recovery time and faster split-root development. The adoption of hydroponics for our method offers several notable advantages, including the formation of well-developed lateral roots, minimization of the risk of pathogen entry through the primary root incision site, space efficiency, rapid root growth due to efficient nutrient delivery, and unobstructed visualization of roots while they grow. The potential application of a standardized split-root system for cotton is vast. It can aid in investigations on plant responses to nutrient availability, nutrient transport, soil-borne pathogens, drought stress, as well as to study the systemic vs. local effects triggered by plant growth-promoting rhizobacteria or mycorrhizal fungi [7].

Limitations

Although the method presented here provides a reproducible framework for split-root experiments in upland cotton under hydroponic conditions, several potential limitations should be acknowledged. First, the scalability of the system to larger experimental designs involving hundreds of plants has not been evaluated, and space, labor, and cost requirements may become limiting factors. Similarly, variety-specific differences under stress treatments cannot be excluded and may influence performance in certain contexts. Second, the method is sensitive to the composition of the nutrient solution (here we used Long Ashton because it is commonly used in our lab), and small changes in formulation could alter plant growth responses; this may require optimization by individual laboratories. Third, the approach relies heavily on controlled hydroponic conditions and sterile handling, which limits its immediate applicability in less controlled environments such as greenhouses. While simplified adaptations may be feasible, their reproducibility remains to be tested. Fourth, some procedural aspects may introduce bias or stress, such as the initial root trimming step, and the requirement for specialized equipment (growth chambers, biosafety cabinets, hydroponic systems) could restrict broader adoption. Taken together, these limitations highlight that this split-root method for cotton is best suited for controlled laboratory studies, and its broader application will depend on the specific research goals and resources available.

Related research article

None

Ethics statements

None

Supplementary material and/or additional information

Table S1. Recipe of the Long Ashton medium used for hydroponics.

Table S2: Comparison of root biomass between compartments A and B across eight cotton varieties (Wilcoxon signed-rank test).

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CRediT authorship contribution statement

Brianna J. Jamison: Conceptualization, Methodology, Investigation, Visualization, Validation, Writing – original draft. **Rekha Pandey:** Conceptualization, Methodology, Investigation, Visualization, Validation, Writing – original draft. **Matheus Morais:** Writing – original draft. **Amanda A. Cardoso:** Conceptualization, Supervision, Writing – review & editing. **Kevin Garcia:** Conceptualization, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.mex.2025.103714.

Data availability

Data will be made available on request.

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