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A Low-volume Hydroponic Protocol for Maize and A Sensitive Bud-length Assay for Root-to-Shoot Impacts of The Strigolactone Analog, *rac*-GR24

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[Abstract] Hydroponic approaches have been a boon for root research by facilitating root-feeding studies as well as secretion analysis. Results have aided our understanding of root-shoot signaling, transport, hormone function and more. However, existing approaches are often restricted to small plants and seedlings by prohibitive cost or availability of experimental substrates. In addition to this, research on the hormone, strigolactone (SL) has been constrained in species like maize by a lack of specific assays for quantifying responses. Here a low-volume hydroponic approach was developed for growing maize plants to the 3-leaf stage (about 2.5 weeks and 12 cm tall at the 2nd leaf collar) using 4 plants with 1 L of aerated media. This protocol also allowed development of an assay for root-to-shoot impacts of the SL analog, *rac*-GR24, which has a prominent, easily-quantifiable effect on outgrowth of lateral buds on maize shoots. Advantages of the protocol include the cost-effective ease and precision of its capacity for quantifying root-to-shoot SL responses. In addition, the low-volume hydroponic approach can be readily adapted for continuous or pulse-type root-feeding studies, root-labeling experiments, and/or secretion sampling in maize and other large, C4 grasses like sorghum, sugarcane, and miscanthus.
Keywords: Strigolactone, Hydroponics, Lateral bud outgrowth, Root-to-shoot transport, Root signals, Root labeling, Root feeding, C4 grasses

[Background] The initial purpose for developing the protocol presented here was to quantify root-to-shoot impacts of the hormone, strigolactone (SL) in maize. A central role was postulated for strigolactone in domestication of this species, due to contributions by this hormone to plant architecture and thus the single-stalk morphology of modern-day maize (Guan *et al.*, 2012). To address this possibility, a system was needed that would 1) allow root-feeding studies throughout initial vegetative growth of this large grass species, 2) minimize amount and cost of the SL analog to be used (*rac*-GR24), and 3) allow accurate quantification of shoot responses in terms of lateral-bud outgrowth. The resulting protocol can be adapted to a wide range of root-feeding, root-labeling, and exudate-sampling studies with low-volume hydroponic growth of large, C4 grass species. A new approach for strigolactone study in these species is also provided.

Strigolactones (SLs) contribute to apical dominance in diverse species (Wang *et al.*, 2018). A similar suppression of lateral shoots is a key domestication feature in maize. The single-stalk architecture results from elevated expression of a domestication gene, *Teosinte branched 1* (*Tb1*) (Doebley *et al.*, 1997). Under normal conditions, lateral buds are tiny, protected by leaf sheaths, and remain dormant in most maize inbreds. The buds do not elongate into visible tillers unless apical dominance is released by

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decapitation or especially favorable growth conditions. The possible role of SLs in this process emerged with their identification as phytohormones involved in axillary branching (Gomez-Roldan *et al.*, 2008; Umehara *et al.*, 2008). Direct contributions in maize were revealed by the transposon insertion into a gene for SL biosynthesis (*Zea mays Carotenoid Cleavage Dioxygenase 8* (*ZmCCD8*). The resulting, SL-deficient maize mutant, *zmccd8*, showed characteristics of a primitive, pre-domestication phenotype that included outgrowth of lateral buds and limited apical dominance (Guan *et al.*, 2012).

Although genes for SL biosynthesis are expressed in many tissues, most of its formation occurs in roots. The root-derived SLs are transported acropetally to shoots through xylem (Kohlen *et al.*, 2010) and via ABC transporters (Sasse *et al.*, 2015). Plant roots also secrete SLs into the rhizosphere, where they attract symbiotic partners such as arbuscular mycorrhizal fungi (Akiyama *et al.*, 2005) and nitrogen-fixing bacteria (López-Ráez *et al.*, 2017). Unfortunately, the SL exudates are also sensed by seeds of parasitic weeds like *Striga* that use these secretions as signals to germinate and locate host roots (Aliche *et al.*, 2020). The discovery of diverse SL functions enhances its potential applications in agriculture (Aliche *et al.*, 2020).

Hydroponic systems have been used to study roles of SLs in axillary branching by *Arabidopsis* (Waters *et al.*, 2012) and rice (Umehara *et al.*, 2008). This approach has also been widely used to investigate homeostasis of mineral nutrients and processes that include iron uptake in maize (Nozoye *et al.*, 2013). However, analysis of SL effects on lateral buds in maize seedlings required improvements in the efficiency of existing hydroponic protocols. Two paramount considerations were 1) the expense of *rac*-GR24, and 2) the quantity needed to support development of lateral buds. The synthetic SL analog, *rac*-G24, is costly as well as short-lived, with a half-life around 3 days in hydroponic solution. Supplies must thus be refreshed every 3 to 4 days, and the hydroponic system must have a low enough solution volume to minimize usage of *rac*-GR24. At the same time, the compact system must also provide enough space for growth of the lateral buds and roots. The bud outgrowth in particular, is sensitive to changes in nutrient availability and growing space. The protocol described here addresses the above requirements. It can also be used to advance other research in the field of root biology, plant-microbe interactions, crop-weed interactions, and mechanisms of mineral-nutrient homeostasis in grass crops.

Materials and Reagents

1. Disposable centrifuge tubes, sterile, polypropylene, calibrated, 50-ml (Fisher Scientific, Fisherbrand, catalog number: 06-443-20)
2. Paper towels (Geogia Pacific, singlefold 23504, 26.035 cm x 23.495 cm)
3. Aluminum foil (Reynolds Kitchens, Reynolds Wrap)
4. Corn kernels (here using wild type [W22 inbred] and a mutant [*zmccd8* in a W22 background])
5. Bleach (8.25% Sodium Hypochloride) (standard grocery or hardware-store type, without surfactants or other additives)
6. *rac*-GR24 ($C_{17}H_{14}O_5$) (Chiralix, catalog number: CX23880-5MG)
7. Acetone (C_3H_6O) (Fisher Scientific, Fisherbrand, catalog number: A949-1)

8. Potassium nitrate (KNO_3) (Sigma-Aldrich, catalog number:P-8394)
9. Calcium nitrate tetrahydrate ($Ca(NO_3)_2·4H_2O$) (Fisher Scientific, Fisherbrand, catalog number: C109-500)
10. Potassium dihydrogen phosphate (KH_2PO_4) (Fisher Scientific, Fisherbrand, catalog number: BP362-1)
11. Magnesium sulfate, anhydrous ($MgSO_4$) (ACROS, catalog number: 7487-88-9)
12. Boric acid (H_3BO_3) (Fisher Scientific, Fisherbrand, catalog number: A73-1)
13. Ethylenediamine tetra acetic acid, Ferric sodium salt ($C_{10}H_{14}N_2O_8FeNa$) (Research Products International, catalog number: E57040-100.0)
14. Molybdenum (VI) oxide (MoO_3) (ACROS, catalog number: 1313-27-5)
15. Ammonium metavanadate (NH_4VO_3) (ACROS, catalog number: 7803-55-6)
16. Manganese (II) sulfate monohydrate ($MnSO_4·H_2O$) (Sigma-Aldrich, catalog number: M7634)
17. Copper (II) sulfate pentahydrate ($CuSO_4·5H_2O$) (Sigma-Aldrich, ACS, catalog number: 209198)
18. Zinc sulfate ($ZnSO_4·7H_2O$) (Sigma-Aldrich, ReagentPlus, catalog number: Z4750-100G)
19. Hydrochloric acid (HCl) (Fisher Scientific, Fisherbrand, catalog number: A144-212)
20. Hydroponic stock (see Recipes)
21. Hydroponic working solution (see Recipes)
22. *rac*-GR24 solution (see Recipes)

Equipment

1. Aeration tubing, standard, 8M (about 25 feet), 4-mm inside diameter (Walmart, Aqua culture, 928/25-S)
2. Aquarium T-way connector valve (Walmart, Aqua culture, PV-T/2)
3. Aquarium bubble stone (Walmart, Aqua culture, ASC1)
4. 1,000-ml glass beaker (Fisher Scientific, PYREX No. 1000)
5. Styrofoam sheets (~1.3 cm thick, white)
6. Empty pipette-tip racks (Rainin, model: GP-L10F, or comparable type)
7. Plastic sheet [M-D 04762 Weatherstrip sheeting, 0.1016 mm (4 mil.) thick, clear; Waltmart]
8. Roto-shaker (Scientific Industries, Roto-Shake Genie)
9. Isotemp Incubator (Fisher Scientific, Fisherbrand)
10. Magnetic stir plate (Barnstead International, Barnstead Thermolyne, catalog number: S131125)
11. Magnetic stir bars (Fisher Scientific, Fisherbrand, catalog number: 22-331447, 9.5 mm x 50.8 mm)
12. Aquarium air pump (Tetra Whisper, Walmart)
13. Walk-in growth chamber (Advanced Intellus environmental controller, Percival, or comparable model)
14. 10-, 50-, 100-, 250-, and 1,000-ml graduated cylinders (Fisher Scientific, Fisherbrand)
15. 2-L graduated plastic beaker

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16. 4-L graduated plastic beaker (Fisher Scientific, Fisherbrand)
17. Pipettes and tips (Fisher Scientific, Fisherbrand)
18. pH meter (pH meter 430) (Corning, catalog number: 476436, or comparable type)
19. Micro-ruler (calibrated to 0.1 mm, 5 mm range) (Ted Pella, catalog number: 13623)
20. Analytical balance (Mettler Toledo, model: AB104-S, or comparable model)
21. Scissors (standard, Walmart)
22. Utility knife (standard, Walmart)
23. Lab scalpel (Feather #3 with surgical design No. 11 carbon scalpel blade, Fisher Scientific, or equivalent)
24. Forceps (Fisher Scientific, Fisherbrand, catalog number:12000122, or equivalent)
25. Stereo microscope (Leica MZ12S or equivalent model)

Procedure

A. Seed/kernel sterilization
   1. Prepare 3% (v/v) sodium hypochlorite by mixing 50-ml bleach with 87.5-ml deionized water.
   2. Put 30 maize kernels into a calibrated 50-ml disposable centrifuge tube.
   3. Add the diluted bleach to a total volume of 40-ml using the calibration scale on the tube.
   4. Fasten the tube securely on a roto-shaker and let it rotate for 10 min at about 25 RPM.
   5. Pour off the bleach solution and rinse kernels thoroughly with running water for 5 min.

B. Seed/kernel germination
   1. Place two paper towels on a plastic sheet (~24 cm x 38 cm).
   2. Wet the paper towels with water and remove any bubbles caught between the towels and plastic sheet.
   3. Line kernels in a row about 5 mm apart and 2 to 3 cm (about 1 inch) from one long edge of the paper towel (this will become the top) (Figure 1A).
   4. Layer two more paper towels on top of the kernels and add water until all paper towels are soaked completely (Figure 1B).
   5. Roll the wet, multi-layer germination bed into a spiral with the plastic sheet to the outside. Final diameter should be approximately 6 cm (Figure 1C).
   6. Prop the germination roll in a secure, upright position inside a container (*e.g.*, 3-L polypropylene beaker) and add enough water to submerge the lower 2-cm of the paper towels.
   7. Cover the container with aluminum foil tightly enough to minimize water loss, but allow air exchange.
   8. Incubate at 24 °C in darkness for 3 to 4 days (Figure 1D).

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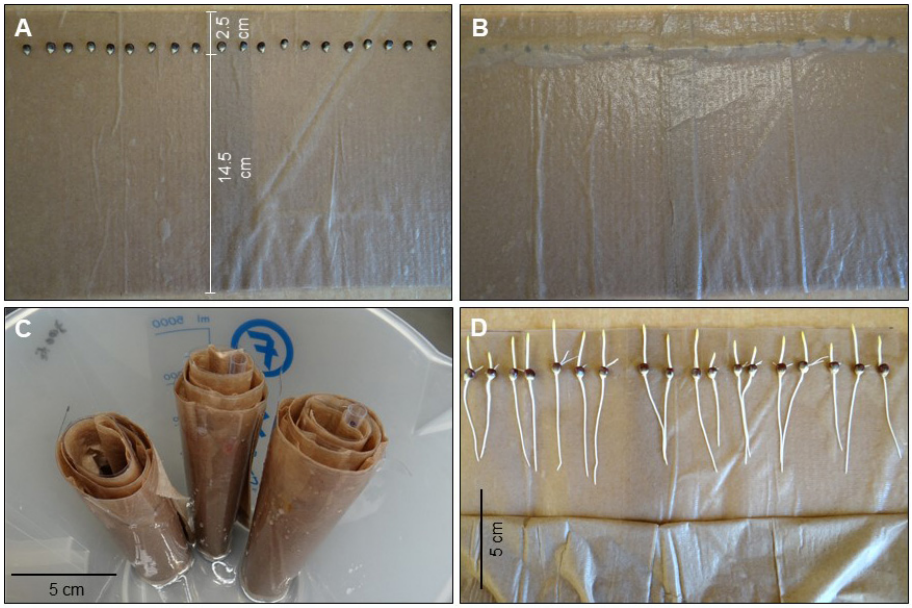

Figure 1. Multi-layer germination rolls. A. Surface-sterilized maize kernels are placed in a line about 2 to 3 cm (1 inch) below one long edge of two wet paper towels. B. The kernels are covered by wet paper towels. C. Prop the germination roll in a secure, upright position inside a container (*e.g.*, 3-L polypropylene beaker) and add enough water to submerge the lower 2-cm of the paper towels. D. 3-day-old etiolated maize seedlings grown in a paper roll.

C. Prepare components of the hydroponics system

1. Use scissors to trim corners from empty plastic pipette-tip racks and adjust size to fit snugly inside a 1-L glass beaker (Figure 2A).
2. Use a utility knife to cut Styrofoam sheets into discs with diameters to fit snugly inside a 1-L glass beaker (diameter = 10.2 cm, Figure 2B).
3. Use a lab scalpel to cut four holes (each about 1-cm in diameter) through the Styrofoam disc. Each hole should be about 1.5 to 2 cm apart.
4. Cut the aeration tubing into short (12-cm) and long (30-cm) lengths [4 shorts and 7 longs for 6 tanks (1-L beakers)].

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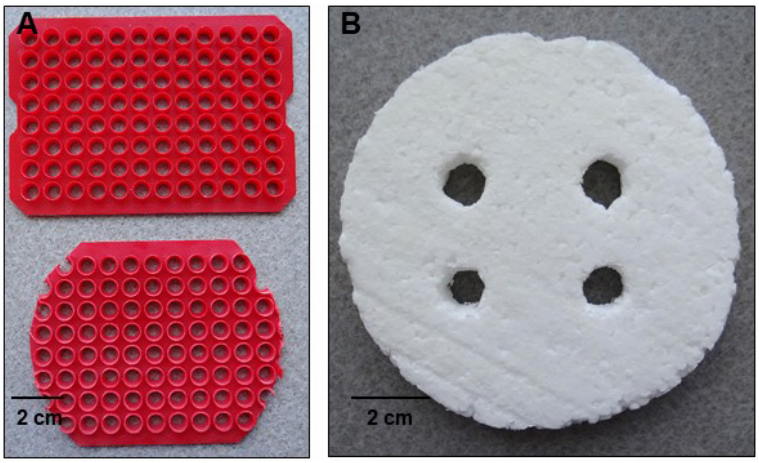

Figure 2. Configuration of plastic tip-rack and Styrofoam disc for holding maize seedlings in nutrient solution. A. The tip rack (bottom) was cut at its corners and size-adjusted to fit snugly into the 1-L glass beaker. B. The Styrofoam disc was similarly adjusted for fit into a 1-L beaker, and 4 small holes added to hold maize seedlings in place (see text for dimensions).

D. Prepare hydroponic solution
1. Fill the 4-L graduated plastic beaker with 3.5-L deionized water.
2. Add a stir bar (at least 5-cm) to the beaker.
3. Place the beaker on a magnetic stirrer and initiate vortexing (6,000 rpm).
4. Use a graduated cylinder to measure 40 ml of the 100x macro-nutrition stock solution A (0.8 M KNO_3).
5. Add this (40 ml stock solution A) to the 4-L beaker.
6. Repeat these two steps for adding 40 ml of the 100x macro-nutrition stocks: Solution B (0.2 M $Ca(NO_3)_2$), solution C (0.2 M KH_2PO_4), and solution D (0.2 M $MgSO_4$).
7. Add 4 ml of the 1,000x Fe-EDTA stock solution into the beaker.
8. Add 4 ml of the 1,000x micro-nutrition stock solution to the 4-L beaker.
9. Fill the beaker with deionized water to 4 L.
10. Adjust pH to 5.5 with 1.0 N HCl.
11. Aliquot 2 L of the final solution to fill a fresh 2-L graduated plastic beaker.

E. Prepare hydroponic solutions with and without *rac*-GR24 (both with 0.001% acetone)
1. Place a fresh 2-L graduated plastic beaker (labeled as GR24) on a stirrer.
2. Add a stir bar at least 5-cm long to the beaker.
3. Fill with 2 L of the prepared hydroponic solution described above (Procedure C).
4. Initiate stirring.
5. For the *rac*-GR24 treatment, pipette 20-µl of 10,000x *rac*-GR24 stock solution into the 2-L graduated plastic beaker.
6. For the control treatment, pipette 20-µl of acetone (100%) into the remaining 2 liters of hydroponic solution in the 4-L graduated plastic beaker.

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F. Assemble the hydroponics system

1. Fill a 1-L glass beaker with 900-ml of hydroponic solution.
2. Attach a bubble stone to the long sections of aeration tubing (30 cm) and insert this into the beaker.
3. Fit the pipette-tip rack inside the 1-L beaker and position it at approximately the d 900-ml level (Figure 3A).
4. Carefully unwrap the 4-day-old maize seedlings from their multilayered roll of germination paper (Figure 1D)
5. Select 4 uniform seedlings from these and thread their roots through holes in the plastic rack so that they're positioned about 2 cm apart (Figure 3B). Their positions should also be aligned with sites of holes in the Styrofoam disc that will sit above them.
6. Carefully fit the shoot of each seedling through one of the holes in the Styrofoam disc, then push down on the disc to seat it about 1.5 cm above the plastic rack holding the seedlings (Figures 3C and 3D).
7. Connect short lengths (12-cm) of aeration tubing with T-way connector valves.
8. Connect longer aeration tubes (30-cm) to each outlet of the T-way connectors.
9. Use a long section of aeration tubing (30-cm) to connect the aerating system with the air pump.
10. Plug the air pump into an electric outlet and activate it.
11. Wrap each beaker with aluminum foil to keep roots in darkness and minimize fluid loss (Figures 3E and 3F).
12. Grow the seedlings in a growth chamber at 24 °C with a photoperiod of 16 h light and 8 h dark for 17 days.
13. Refresh the hydroponic solution every 4 days.

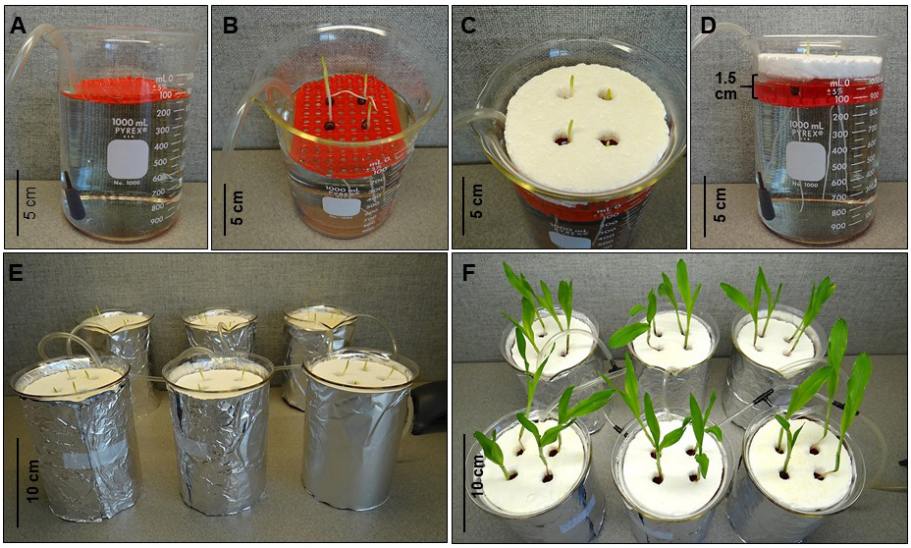

Figure 3 Assembly of the hydroponic culture system. A. Glass beaker with a bubble stone and tip rack. B. Maize seedlings positioned on the plastic tip rack. C. Maize seedlings held upright with support from the Styrofoam disk. D. Side view of the hydroponic beaker. E. The

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hydroponic beakers are covered with aluminum foil to keep roots in darkness and minimize fluid loss. F. Maize seedlings after 5 days in hydroponic solution.

G. Measurement of lateral bud length

1. Use scissors or utility knife to cut shoots from the 3-week-old seedlings at the base of the mesocotyl (Figure 4A).
2. Use a scalpel and forceps to carefully remove the coleoptile and sheath of the first leaf (Figure 4B).
3. Measure the lateral bud length with a Micro-ruler under a stereo microscope (Figure 4C).

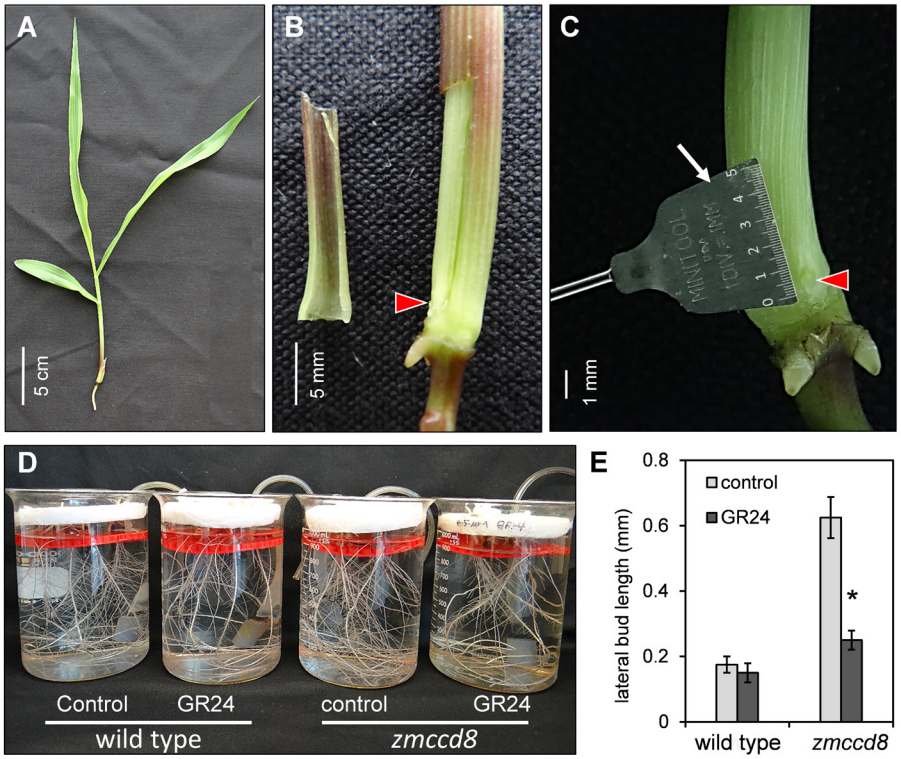

Figure 4. Dissection and measurement of lateral bud length. A. Shoot of a 17-day-old maize seedling from the low-volume hydroponic culture system. B. Dissection of the first leaf sheath to reveal the lateral bud (red arrowhead). C. Measuring the length of lateral bud (red arrowhead) with a 5-mm Micro-ruler (white arrow). D. Root morphology of the 17-day-old maize seedlings in the low-volume hydroponic culture. E. Statistical analysis of the lateral bud length with or without GR24 treatment. The asterisk indicate significant difference between control and GR24 treatment in *zmmcd8* mutants ($P < 0.01$, n = 4).

Data analysis

Statistical analysis was done with AVONA in Excel (Microsoft).

Recipes

1. Hydroponic stock

Macronutrients	MW (g/mol)	100x stock (M)	Weight for 1-L stock (g)	Volume of stock for 1-L working nutrient solution (ml)	Final concentration (mM)
KNO_3	101.1	0.8	80.88	10.00	8.00
$Ca(NO_3)_2{\cdot}4H_2O$	236.1	0.2	47.22	10.00	2.00
KH_2PO_4	136.1	0.2	27.22	10.00	2.00
$MgSO_4$	120.37	0.2	24.074	10.00	2.00

2. Hydroponic working solution

Micronutrients	MW (g/mol)	1,000x stock (mM)	Weight for 1-L stock (g)	Volume of stock for 1-L working nutrient solution (ml)	Final concentration (μM)
NaFe(III)EDTA	367.1	100	3.671	1.00	100.00
H_3BO_3	61.83	46	2.84	1.00	46.00
$MnSO_4{\cdot}H_2O$	169.0	10	1.69	1.00	10.00
$CuSO_4{\cdot}5H_2O$	249.68	0.32	0.08	1.00	3.20
$ZnSO_4{\cdot}7H_2O$	287.5	0.77	0.22	1.00	0.77
MoO_3	143.94	0.58	0.08	1.00	0.58
NH_4VO_3	116.98	0.25	0.03	1.00	0.25

3. *rac*-GR24 solution

***rac*-GR24 (keep at -20 °C)**	**MW (g/mol)**	**10,000x stock in acetone (mM)**	**Weight for 168-μl stock (mg)**	**Volume of stock for 1-L working nutrient solution (μl)**	**Final concentration (μM)**
$C_{17}H_{14}O_5$	298.29	100	5.00	10.00	1.00

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letting us use the Micro-ruler. The authors declare no competing interests in regard to this publication.

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