ISSN: 2690-912X

Research Article Journal of Genetic Engineering and Biotechnology Research

Hydroponic Cultivation of *Nicotiana benthamiana* for Biomass Production in Molecular Farming

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Submitted: 2026, Oct 01; **Accepted:** 2026, Nov 03; **Published:** 2026, Nov 17

Citation: Deo, P. C. (2026). Hydroponic Cultivation of Nicotiana benthamiana for Biomass Production in Molecular Farming. *J Gene Engg Bio Res, 8*(3). 01-10.

Abstract

This protocol outlines an efficient hydroponic system for cultivating Nicotiana benthamiana plants as a platform for recombinant protein production. Two hydroponic systems, Deep Water Culture (DWC) and Ebb and Flow (E & F), were compared with conventional tissue culture methods, focusing on plant growth and leaf biomass accumulation. The finding show that hydroponic cultivation yields a ~17-fold increase in leaf biomass per plant compared to tissue culture, with no significant difference in yield between DWC and E&F systems. However, the E&F system offers improved aeration and reduced risk of root diseases, making it more suitable for long-term cultivation. This scalable cultivation pipeline, when combined with stable genetic transformation and clonal propagation via tissue culture, provides a robust and sustainable strategy for high-yield molecular farming using N. benthamiana.

Keywords: *Nicotiana benthamiana*, Hydroponics, Ebb and Flow, Deep Water Culture, Recombinant Protein Production, Tissue Culture, Molecular Farming, Plant-Based Bioproduction, Transgenic Plants, Biomass Accumulation

1. Introduction

Nicotiana benthamiana is a fast-growing, herbaceous plant native to Australia that has become a model species in molecular farming due to its amenability to *Agrobacterium*-mediated transformation, high recombinant protein expression, and ease of cultivation [1,2]. It produces fewer interfering secondary metabolites than other tobacco species, such as *N. tabacum*, making downstream purification of proteins simpler and more cost-effective. These traits have positioned *N. benthamiana* as a reliable platform for the transient expression of high-value proteins, including vaccine candidates, enzymes, and antibodies [3,4].

Micropropagation of *N. benthamiana* through axillary shoot proliferation has been demonstrated as an efficient method for producing clean, uniform planting material [5,6]. However, while tissue culture can yield significant biomass, it is labour-intensive and resource-demanding, limiting its scalability for industrial recombinant protein production.

Hydroponic cultivation offers a scalable, cost-effective alternative, supporting consistent biomass production under controlled conditions. In hydroponic systems, plants are grown in inert substrates such as hydroton or rockwool and receive nutrient-rich solutions either continuously or intermittently [7]. These systems have been extensively applied in food and ornamental crop production and are increasingly being adapted for non-food crops used in research and biomanufacturing [8,9]. Hydroponic systems offer several advantages: continuous biomass production year-round, reduced risk of soil-borne diseases, enhanced growth through direct nutrient availability, optimized space usage and environmental control, uniform plant development for synchronized harvesting. Beyond production, hydroponic platforms are also useful for controlled stress experiments in plant research, including nutrient deficiency, salinity, and heavy metal tolerance studies [10,11].

This paper outlines a step-by-step method for cultivating tissue-culture-derived *N. benthamiana* plants in hydroponic systems, with an emphasis on two commonly used setups: Deep Water Culture (DWC) and Ebb and Flow (E & F) (Figure. 1).

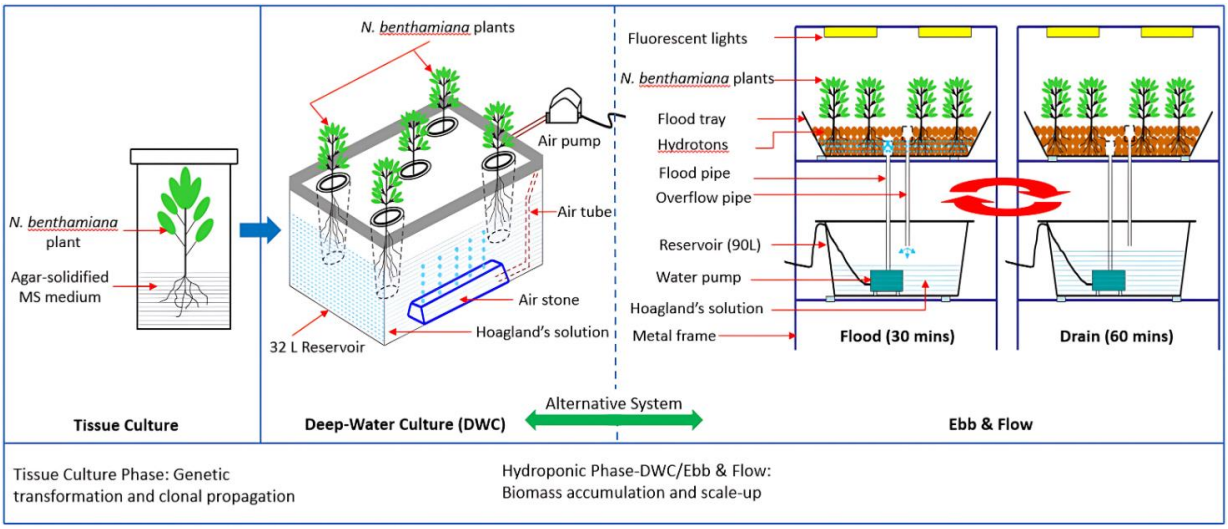


Figure 1: Integrated cultivation pipeline for high biomass production in *Nicotiana benthamiana*. Tissue culture provides the foundational platform for genetic transformation and clonal propagation of wild type and transgenic lines. Regenerated plantlets are transferred to hydroponic systems: deep-water-culture or ebb & flow for biomass accumulation and scale-up. The ebb & flow is recommended for long-term cultivation due to improved oxygenation, dynamic nutrient flow, and reduced risk of root pathogens such as *Pythium*. This figure was generated in Microsoft PowerPoint

2. Materials and Methods

2.1. Experimental Design and Growth Conditions

Three independent experiments were conducted at different time periods to evaluate *Nicotiana benthamiana* propagation and cultivation using tissue culture (TC), deep-water culture (DWC) and ebb-and-flow (E&F) hydroponic systems. For TC, 60, 70 and 60 plants were used in the first, second and third experiments, respectively, giving a cumulative total of 190 plants. For DWC, 10 plants were used in each of the first two experiments and five plants in the third experiment because of root rot problems encountered during the study, giving a cumulative total of 25 plants. Each DWC container accommodated a maximum of five plants. For E&F cultivation, 24, 22 and 22 plants were used in the first, second and third experiments, respectively, giving a cumulative total of 68 plants.

3. Preparation of Culture Media and Nutrient Solutions

3.1. Shoot Induction Medium

Shoot induction medium was prepared using Murashige and Skoog (MS) basal medium powder at 4.3 g L^{-1}, supplemented with sucrose at 30 g L^{-1} and agar at 7 g L^{-1}. The pH was adjusted to 5.8 using 1 M NaOH, and the volume was made up to 1 L with Milli-Q water. The medium was autoclaved at 121 °C and 15 psi for 15 min. After autoclaving, the medium was allowed to cool to 40–50 °C before kinetin (0.5 mg L^{-1}) and indole-3-butyric acid (IBA; 0.1 mg L^{-1}) were added. The supplemented medium was mixed gently and dispensed at 20 mL per sterile 120 mL culture vessel.

3.2. Plant Development Medium

Plant development medium was prepared using Murashige and Skoog (MS) basal medium powder at 4.3 g L^{-1}, supplemented with sucrose at 30 g L^{-1} and agar at 7 g L^{-1}. The pH was adjusted to 5.8 using 1 M NaOH, and the volume was made up to 1 L with Milli-Q water. The medium was autoclaved at 121 °C and 15 psi for 15 min. After cooling to 40–50 °C, the hormone-free medium was aseptically dispensed at approximately 100 mL per sterile 500 mL culture vessel under laminar airflow conditions.

3.3. Hydroponic Nutrient Medium

Half-strength Hoagland's nutrient solution was prepared from concentrated macronutrient, micronutrient and iron–EDTA stock solutions, which were prepared in Milli-Q water and stored at 4 °C until use. MES buffer was added to a final concentration of 0.5 mM to stabilise the pH. The solution was initially adjusted to pH 6.0 before MES addition, resulting in a final pH of approximately 5.6–5.7. The nutrient solution was then made up to a final volume of 20 L for the deep-water culture system or 60 L for the ebb-and-flow system using tap water. Detailed stock solution formulations and preparation procedures are provided in Supplementary Methods S1.

3.4. Plant Material and in Vitro Propagation

Nicotiana benthamiana plants were propagated in vitro using nodal cuttings, following the method described by Deo [6]. Axillary shoots were initiated on the shoot induction medium described above, consisting of Murashige and Skoog (MS) basal medium supplemented with kinetin (0.5 mg L^{-1}) and indole-3-butyric acid (IBA; 0.1 mg L^{-1}).

For root development, shoots were transferred either to liquid plant development medium (20 mL) supported by filter-paper bridges in 50 mL polycarbonate culture vessels or to agar-solidified plant development medium in 500 mL culture vessels. Both media contained MS basal medium powder at 4.3 g L^{-1} and sucrose at 30 g L^{-1}, with agar (7 g L^{-1}) included only in the solid medium. The pH was adjusted to 5.8 before autoclaving at 121 °C and 15 psi

for 15 min. Cultures were maintained for four weeks under a 16 h photoperiod at 25 °C and a photon flux density of 30 µmol m^{-2} s^{-1}.

Plantlets exceeding 5 cm in height and possessing well-developed roots were selected for hydroponic cultivation. Before transplantation, plantlets were removed from the culture vessels, and their roots were thoroughly washed under running tap water and temporarily immersed in clean tap water.

3.5. Preparation of the Hydroponic Growing Medium

Lightweight expanded clay aggregate (Hydroton; LECA) was used as the growing medium. Before use, the aggregate was washed thoroughly under running tap water to remove dust and debris, followed by autoclaving at 121 °C and 15 psi for 45 min. The sterilised aggregate was used to support plantlets in the hydroponic systems.

3.6. Hydroponic Cultivation Systems

Two hydroponic cultivation systems were established: deep-water culture (DWC) and ebb-and-flow (E&F).

3.7. Deep-Water Culture

The DWC system consisted of a 32 L plastic container fitted with a lid and filled with 20 L of half-strength Hoagland's nutrient solution. Five sterile 10 cm polyethylene pots containing sterilised Hydroton were positioned in openings in the lid, with one plantlet transplanted into each pot. The nutrient solution was continuously aerated using an air pump connected to tubing. Individual plants were covered with polyethylene bags to maintain humidity following transplantation. The system was maintained at 24 °C under a 16 h light/8 h dark cycle, with a photon flux density of 60–70 µmol m^{-2} s^{-1} provided by cool-white, fluorescent lighting and a relative humidity of 60-70%.

3.8. Ebb-and-Flow System

The E&F system consisted of a 1 m × 1 m plastic tray containing a 7 cm layer of sterilised Hydroton, connected to a 90 L black polyethylene reservoir containing 60 L of half-strength Hoagland's nutrient solution. Tissue-cultured plantlets were transplanted directly into the growing medium. Nutrient solution was pumped into the tray for 30 min and then drained for 60 min. This flood-and-drain cycle was repeated throughout the 16 h light period, with the nutrient solution maintained at a depth of approximately 3 cm during flooding.

Following transplantation, individual plants were covered with polyethylene bags for two weeks to maintain humidity. Temperature, lighting and humidity conditions were maintained as described for the DWC system.

3.9. Nutrient Solution Management and System Maintenance

The nutrient solution was replaced every two weeks. Before refilling, reservoirs were cleaned with 70% ethanol, and tap water was circulated through the system for 2–3 h to flush residual salts. Fresh nutrient solution was then added. During DWC system cleaning, Pythoff™ (0.5 mL L^{-1}) or 3% hydrogen peroxide (1 mL L^{-1}) was used as a contamination-control treatment, as specified in the protocol.

Both systems were inspected every two days to assess nutrient solution levels and signs of microbial contamination. The initial water level was marked when each reservoir was filled with nutrient solution. Water levels were checked against this mark, and any decrease below the mark was corrected by adding clean tap water only to replace water lost through evaporation.

3.10. Leaf Harvesting and Fresh Biomass Determination

Leaves were harvested from the hydroponically grown plants eight weeks after transplantation. Harvested leaves were immediately transferred into pre-weighed polyethylene bags and kept on ice during handling to minimise desiccation. Total leaf fresh weight was determined by weighing each bag containing the harvested tissue and subtracting the corresponding bag tare weight. Mean fresh weight per plant was calculated by dividing the total fresh weight by the number of plants harvested.

Following fresh-weight determination, leaf samples were stored at −80 °C until further use for total soluble protein extraction and purification.

4. Results and Discussions

Axillary shoots of *N. benthamiana* cultured on both filter paper bridges immersed in liquid MS medium and on agar-solidified MS medium initiated root development by Day 7. By Week 4, the regenerated plantlets were usually over 5 cm tall and exhibited a well-developed root system (Figure. 2). Agar-solidified medium supported robust root and shoot development by providing stable physical support and consistent nutrient availability, which helps maintain plantlet morphology and reduces hyperhydricity often observed in liquid cultures [12].

Additionally, agar limits excessive water uptake, promoting stronger cell wall development and improving acclimatization potential after transfer. In contrast, liquid media with filter paper bridges enhanced nutrient and oxygen availability to roots, facilitating faster root elongation and shoot growth due to improved mass transfer and gas exchange [13,12]. However, while the filter paper bridge system was effective for early-stage propagation and experimental setups, it posed practical challenges for large-scale plant production. The fragility of the paper, difficulty in handling numerous plantlets simultaneously, increased risk of microbial contamination and high incidences of vitrification in liquid media make it less scalable and more labour-intensive compared to agar-solidified media or direct transfer methods [14,15].



Figure 2: Rooting in *N. benthamiana* plants derived from axillary shoots growing on "filter paper bridge" in liquid MS (A) and agar-solidified MS (B) ready for hydroponic culture.

When transferred to hydroponic culture, plants began forming new roots within two weeks. Growth during the initial acclimatization phase was slow, but once the humidity cover was removed, a noticeable increase in growth was observed. Existing leaves expanded, and both new roots and shoots emerged (Figure. 3). It is likely that by the time the humidity cover was removed, the plants had developed a sufficient root network to facilitate efficient nutrient uptake and assimilation, resulting in a rapid growth response. Similar observations have been reported in hydroponically grown species, where enhanced root development prior to full exposure improves nutrient absorption and promotes shoot expansion [16,17]. Further, a balanced nutrient solution promotes healthy plant growth, enhances overall plant health, and increases productivity [18].

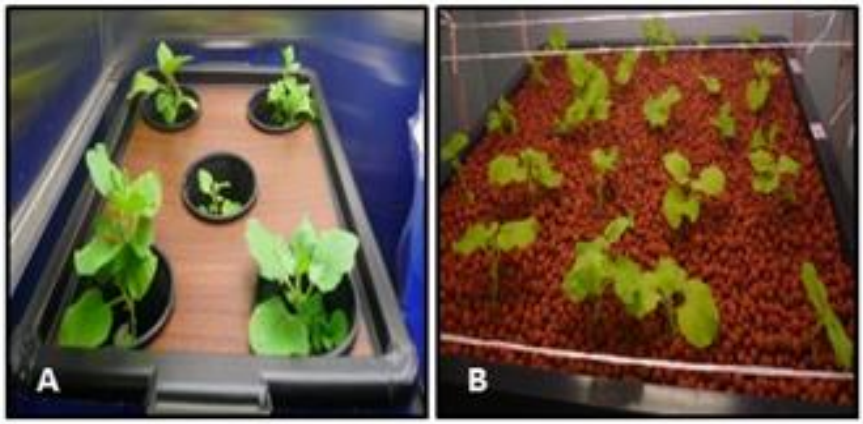


Figure 3: *N. benthamiana* plants at early stages growing in hydroponics system: deep-water culture (A) and ebb & flow (B)

Therefore, to maintain optimal nutrient balance for maximum plant growth and biomass yield, and to minimize microbial contamination during hydroponic cultivation, it was essential to deep-clean the reservoir and replace the nutrient solution every two weeks. The growing medium (hydroton) was also cleaned and sterilized at the end of each growth cycle and before the start of new planting, as recommended by other researchers [19]. The use of filtration systems has been suggested as a means to recycle nutrients and improve water-use efficiency in hydroponics while maintaining sterility [10]. However, implementing such a system was beyond the scope of this study.

By Week 6, plants grown hydroponically developed large leaves measuring approximately 14 × 13 cm — a 3.5-fold increase compared to the original tissue culture-derived leaves (4 × 3 cm) (Figure. 4). The average fresh weight of leaves per plant in both hydroponic systems was approximately 22 g, representing a more than 15-fold increase compared to tissue culture-grown plants (1.3 ± 0.07 g) (Table 1).

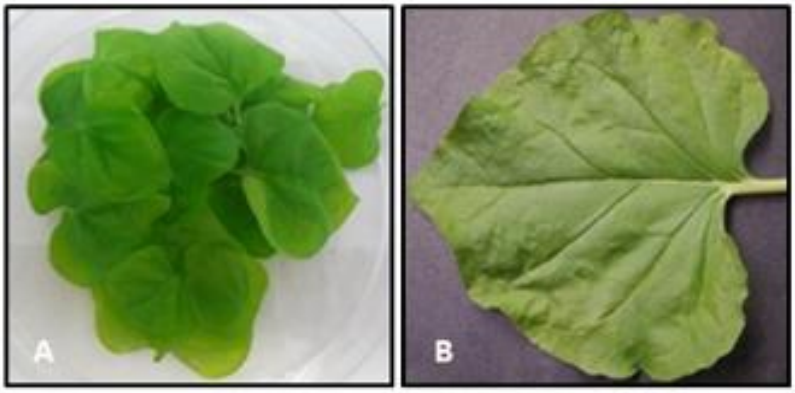


Figure 4: Comparison of the sizes of detached leaves of *N. benthamiana* plants derived from tissue cultured plants (A) and hydroponics (B).

Notably, there was no significant difference in mean biomass between the two hydroponic systems — deep water culture (DWC) (22.8 ± 4.3 g) and ebb and flow (E & F) (22.0 ± 1.0 g) (Figure. 5; Table 1). Despite variation in the number of plants grown per system, hydroponically cultivated plants consistently yielded significantly greater leaf biomass per plant than *in vitro*-grown plants. When extrapolated to the same plant number used in tissue culture (n = 190), the total fresh weight from the E & F system would exceed a 4-fold higher biomass output, underscoring its scalability for biomass-driven applications. These findings highlight the advantages of hydroponic systems in general, and ebb-and-flow (E&F) systems in particular, for molecular farming, especially in contexts where uniform plant growth, nutrient efficiency, and disease mitigation are critical [20]. In addition to high biomass production, hydroponically grown tomatoes contain three times more total soluble proteins (TSP) than conventionally grown greenhouse plants [21]. Based on this, it may be hypothesised that *N. benthamiana* cultivated under hydroponic conditions could produce significantly higher levels of TSP, a trait highly advantageous for molecular farming.

After 4–6 weeks in the DWC system, symptoms of *Pythium* contamination were occasionally observed. Affected plants exhibited slimy roots, wilting leaves, and a foul odour in the nutrient solution (Figure. 6), consistent with previously reported waterborne root pathogens in hydroponic systems [22,23].



Figure 5: Hydroponics culture of *Nicotiana benthamiana*. Six weeks old plants growing in Deep-Water Culture (A) and Ebb & Flow (B)

Table 1: Effect of hydroponics on leaf fresh weight in *Nicotiana benthamiana*

Plant Culture System	Total number of plants cultured	Total fresh weight of leaves (g)	Mean fresh weight of leaves per plant (g)
Tissue culture	190	251.3	1.3 ± 0.07 [b]
Deep water culture	25	486.9	22.8 ± 4.3 [a]
Ebb and flow	68	1499.7	22.0 ± 1.0 [a]

Values represent means ± SEM derived from three independent cultures for each system. Means followed by the same letter are not significantly different ($p < 0.05$), based on one-way ANOVA performed in Microsoft Excel.



Figure 6: *Nicotiana benthamiana* growing in deep-water culture showing symptoms of *Pythium* root rot

Initial treatment with 3% hydrogen peroxide at 1 mL/L provided short-term relief but was limited by the chemical's instability and rapid decomposition. A more consistent and effective treatment involved the application of Pythoff™ (10 g/L monochloramine) at a concentration of 0.5 mL/L. Although treated roots remained slightly brown in appearance, those treated with Pythoff™ maintained better overall health and root density compared to peroxide-treated plants (Figure. 7). These results reinforce the importance of water quality management and pathogen control in hydroponic systems for sustained high productivity [24].

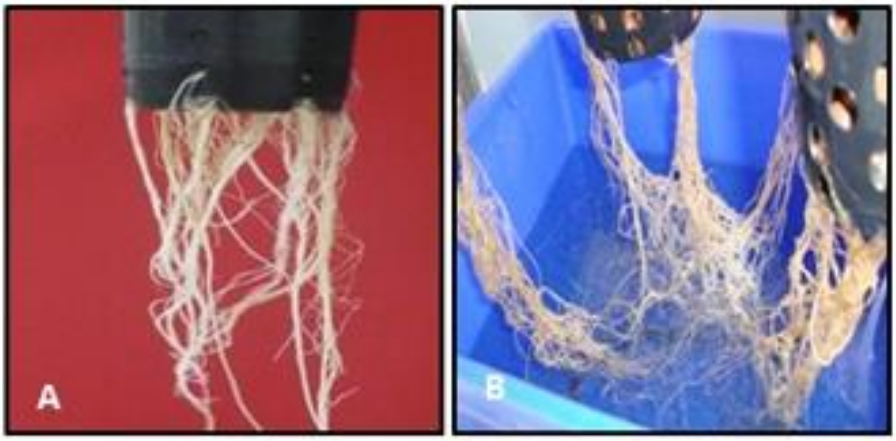


Figure 7: *Nicotiana benthamiana* forming roots in hydroponics with white healthy roots grown in nutrient solution spiked with hydrogen peroxide (A) and Pythoff™ (B)

Although deep water culture (DWC) systems achieved high per-plant biomass, they posed a higher risk of root diseases such as *Pythium* root rot [22]. Despite the use of air pumps for oxygenation, the nutrient solution in DWC systems remains largely static, creating a favourable environment for waterborne pathogens [23]. In contrast, the ebb and flow (E & F) system features periodic wet-dry cycles and media agitation through regular flooding and drainage. This not only enhances oxygenation at the root zone but also significantly reduces the risk of anaerobic conditions and pathogen build-up, including *Pythium* spp [25].

Furthermore, roots in DWC remain continuously submerged, which can lead to hypoxia and tissue softening, increasing susceptibility to root rot [26]. In contrast, E & F systems intermittently submerge the roots, mimicking natural soil moisture dynamics, promoting root resilience, and ensuring systemic aeration with each cycle. These factors make E&F more favourable for long-term cultivation and for reducing crop loss in plant-based protein production pipelines.

While hydroponic systems demonstrated significantly greater biomass output than tissue culture, it is important to emphasize that tissue culture remains the cornerstone of this production system. The generation of transgenic *N. benthamiana* plants, whether via *Agrobacterium*-mediated transformation or other stable transformation methods, relies entirely on *in vitro* regeneration and selection [27]. Moreover, once high-expressing lines are identified, tissue culture provides a clean, pathogen-free method to bulk up

plantlets for subsequent transfer into hydroponic systems.

This creates a robust, modular pipeline in which tissue culture serves as the entry point for genetic engineering and clonal propagation, while hydroponics functions as the downstream engine for biomass and protein production. Recognizing the synergy between these two systems is essential for improving the efficiency and scalability of plant-based recombinant protein production [28].

This hydroponic cultivation protocol enables significantly higher leaf biomass accumulation in *N. benthamiana*, laying a strong foundation for improved recombinant protein yields. While transient agroinfiltration remains a common method, its labour intensity and inconsistent transformation efficiency present limitations for large-scale molecular farming.

Stably transformed lines grown under hydroponic conditions offer a more reliable, continuous, and productive alternative. By addressing the upstream challenge of biomass generation, our system supports a shift toward scalable, high-yield molecular farming. Together, tissue culture and hydroponics form an integrated pipeline from transformation to large-scale cultivation, thus supporting sustainable and efficient recombinant protein production in *N. benthamiana*.

Acknowledgments

This experiment was conducted in the Centre for Tropical Crops and Bio commodities (CTCB) at Queensland University of Technology (QUT) with financial support from Leaf Energy Pty Ltd. The author independently developed the protocol and prepared the manuscript. Special thanks to Prof. James Dale for his support in this work and Prof. Rob Harding for critically reviewing the manuscript.

Supplementary Methods S1: Preparation of Hoagland's Nutrient Stock Solutions

S1.1. Reagents and Stock Solution Preparation

- The stock solutions are prepared as concentrated solutions (e.g., 10X) based on Hoagland and Arnon [7]. Only small volumes are used from these stocks when making the final hydroponic media.
- For the specific amounts of reagents, please refer to the recipe table for each individual stock solution.
- If your turnover is high, it is recommended to prepare stock solutions in 2 L volumes. In such cases, double the quantity of each reagent to maintain the correct concentration.

1. Initial Setup

- In a 1 L beaker, add approximately 800 mL of distilled water.

2. Dissolving Reagents

- Weigh and add the specified amount of each reagent one at a time, stirring continuously until fully dissolved.

3. Make Up to Volume

- Once all components have dissolved, top up the solution to 1 L with distilled water.

4. Storage

- Transfer the completed solution to a clean 1 L Schott bottle.
- Label the bottle clearly with the solution name, concentration, and date of preparation.
- Store at 4°C. These stock solutions can be stored under these conditions for 4–6 weeks without significant loss of efficacy.

5. Before Use

- Always bring the solution to room temperature before use.
- Mix well before preparing the media as salts may settle during storage.

S1.2. Macronutrient Stock Solutions

Stock 1 (Macro Nutrients 1A) - Use 3 mL/L

Reagents	Quantity or Volume	Final Concentration
KNO_3 (1M)	101.1 g	3 mM
$NH_4H_2PO_4$ (0.165M)	18.99 g	0.5 mM
$MgSO_4.7H_2O$ (0.33M)	81.31 g	1.0 mM
Distilled water	Up to 1000 mL	n/a
Total	1000 mL	

Notes:

- KNO_3 provides nitrate (NO_3^-), essential for vegetative growth.
- $NH_4H_2PO_4$ offers both ammonium (NH_4^+) and phosphate ($H_2PO_4^-$), supporting energy metabolism and buffering capacity.
- $MgSO_4 \cdot 7H_2O$ is a source of magnesium, important for chlorophyll and enzyme activity.

Stock 2 (Macro Nutrient 1B) - use 2 mL/L

Reagents	Quantity or Volume	Final Concentration
Ca $(NO_3)_2.4H_2O$ (1M)	236.2 g	2 mM
Distilled water	Up to 1000 mL	n/a
Total	1000 mL	

Notes:

- Ca $(NO_3)_2 \cdot 4H_2O$ supplies both calcium (Ca^{2+}) and nitrate (NO_3^-).
- Avoid mixing with phosphate-rich solutions directly to prevent precipitation.
- Store in dark, if possible, as nitrate is light-sensitive.

Optional Macro Stocks (Alternative Formulation)

Stock 1 (Macro Nutrients 2A) – Use 2.5 mL/L

Reagents	Quantity or Volume	Final Concentration
KNO_3 (1M)	101.1 g	2.5 mM
KH_2PO_4 (0.2M)	27.22 g	0.5 mM
$MgSO_4.7H_2O$ (0.4M)	98.62 g	1.0 mM
Distilled water	Up to 1000 mL	n/a
Total	1000 mL	

Notes:

- Suitable for ammonium-sensitive species or stages.
- KH_2PO_4 offers potassium and phosphate in a more stable form.
- Watch for precipitation if mixed with calcium-containing solutions.

Stock 2 (Macro Nutrient 2B) - use 2.5 mL/L

Reagents	Quantity or Volume	Final Concentration
Ca $(NO_3)_2.4H_2O$ (1M)	236.2 g	2.5 mM
Distilled water	Up to 1000 mL	n/a
Total	1000 mL	

Notes:

- Same as Macro 1B but adjusted for different macro balance.
- Use as a pair with Stock 2A for consistent results.

S1.3. Micronutrient and Iron–EDTA Stock Solutions

Stock 3 (Micro nutrients) - use 0.5 mL/L

Reagents	Quantity or Volume	Final Concentration
H_3BO_3 (50 mM)	2.86 g	25 µM
$MnCl_2.4H_2O$ (10 mM)	1.81 g	5.0 µM
$ZnSO_4.7H_2O$ (0.8 mM)	0.22 g	0.4 µM
$CuSO_4.5H_2O$ (0.3 mM)	0.08 g	0.15 µM
$Na_2MoO_4.2H_2O$ (0.12mM)	0.03 g	0.06 µM
Distilled water	Up to 1000 mL	n/a
Total	1000 mL	

Notes:

- Each micronutrient plays a unique role in metabolism and growth regulation.
- Make sure all components are fully dissolved before use.
- This solution is prone to degradation, so store tightly sealed and in the dark.

Stock 4 (Na_2FeEDTA) - use 2.5 mL/L

Reagents	Quantity or Volume	Final Concentration
$FeSO_4.7H_2O$ (10mM)	2.78 g	25 µM
$Na_2EDTA.2H_2O$ (10 mM)	3.72 g	25 µM
Distilled water	Up to 1000 mL	n/a
Total	1000 mL	

Notes:

Start with Na_2EDTA:

- Add about 800 mL of distilled water to a beaker.
- Add 3.72 g of $Na_2EDTA \cdot 2H_2O$.
- Heat the solution to about 60–70°C (not boiling).
- Stir until the solution becomes clear.

Add $FeSO_4 \cdot 7H_2O$ (Carefully!):

- Weigh 2.78 g of $FeSO_4 \cdot 7H_2O$.
- Slowly add it to the warm EDTA solution while stirring.
- The solution will turn reddish or brown – this is normal.

Top Up and Store:

- Once everything is dissolved and clear, top up the solution to 1 L with distilled water.
- Pour it into a dark bottle and label clearly.
- Store in the fridge at 4°C.

S1.4. MES Buffer Preparation

Stock 5 (MES Buffer) - use 2.5 mL/L

Reagents	Quantity or Volume	Final Concentration
MES (0.2M)	39.06 g	0.5 mM
NaOH (10 M)	To pH 6.0	
Distilled water	Up to 1000 mL	n/a
Total	1000 mL	

Notes:

- MES is effective in the pH range of 5.5–6.7.
- Adjust pH to exactly 6.0 using NaOH before making up to volume.
- Always bring to room temperature before pH adjustment and mixing.

S1.5. Preparation of the Final Hydroponic Nutrient Solution and Growing Medium

Note: Two alternative formulations can be used to prepare the final hydroponic nutrient solution for plant growth:

- **Formulation 1 (used in this research):** Macro stock solutions 1A and 1B, micronutrient stock solution 3, $Na_2FeEDTA$ stock solution 4, and MES buffer stock solution 5.
- **Formulation 2 (alternative):** Macro stock solutions 2A and 2B, micronutrient stock solution 3, $Na_2FeEDTA$ stock solution 4, and MES buffer stock solution 5.

The appropriate stock solutions should be selected according to the desired formulation.

A. Hydroponic Nutrient Solution

- Pipette the appropriate volumes of Hoagland's nutrient stock solutions required to prepare half-strength nutrient solution for the respective hydroponic system:
- Adjust the pH of the combined nutrient stocks to 6.0 and make up the volume to 2 L with tap water.
- Transfer the prepared 2 L nutrient mixture into a clean 2 L Schott bottle, seal and store at room temperature until use.
- Prepare the final nutrient solution in the respective reservoirs by adding the 2 L nutrient mixture to:
- 58 L of tap water for the E&F system (final volume: 60 L).
- 18 L of tap water for the DWC system (final volume: 20 L).
- Add MES buffer (from a 0.2 M stock solution) to the prepared nutrient solution to achieve a final concentration of 0.5 mM (50 mL for DWC and 150 mL for E&F) to stabilise the pH.

B. Preparation of Growing Medium

- Hydroton contains a significant amount of dust and debris. Before use, wash thoroughly under running tap water until the water runs clear.
- Autoclave the cleaned Hydroton at 121 °C and 15 psi for 45 minutes.
- After use, rinse thoroughly with water to remove any residues and allow to air-dry completely before storage or reuse.

References

1. Van Herpen, T. W. J. M., Cankar, K., Nogueira, M., Bosch, D., Bouwmeester, H. J., & Beekwilder, J. (2010). Nicotiana benthamiana as a production platform for artemisinin precursors. *PLOS ONE, 5*(12), e14222.
2. Goodin, M. M., Zaitlin, D., Naidu, R. A., & Lommel, S. A. (2008). *Nicotiana benthamiana*: its history and future as a model for plant–pathogen interactions. *Molecular Plant-Microbe Interactions, 21*(8), 1015–1026.
3. Lumlertdacha B, Mahong B, Rattanapisit K, Bulaon CJI, Hemachudha T, Phoolcharoen W. (2023). Efficiency Comparative Approach of Plant-Produced Monoclonal

Antibodies against Rabies Virus Infection. Vaccines (Basel), 11(8):1377.
4. Park, Y., Kang, H., Min, K., Kim, N.H., Park, M., Ouh, I., Lee, S. (2021). Rabies virus glycoprotein produced in Nicotiana benthamiana is an immunogenic antigen in mice. Czech Journal of Genetics and Plant Breeding, 57(1), 26-35.
5. Deo, P. C. (2025). Practical guide to in vitro clonal propagation of Nicotiana benthamiana using axillary shoot induction. Bio-protocol 15(18): e5443.
6. Deo, P.C., Dugdale, B., Harding, R.M., Kato, M. and Dale, J. (2014). In vitro micropropagation of Nicotiana benthamiana via axillary shoots. The South Pacific Journal of Natural and Applied Sciences 32: 55-60.
7. Hoagland, D. R., & Arnon, D. I. (1950). The water-culture method for growing plants without soil. Circular. *California Agricultural Experiment Station, 347*.
8. Giri, L., Angmo, J. C., Hussain, M., Singh, B., Bhatt, I. D., Nautiyal, S. (2025). Hydroponic culture improves growth and secondary metabolite production in *Rheum tibeticum*, a near threatened species from the Ladakh Trans-Himalayan region of India. Plant Biosystems - An International Journal Dealing with All Aspects of Plant Biology, 159(2), 356–368.
9. Rajaseger, G., Chan, K. L., Tan, K. Y., Ramasamy, S., Khin, M. C., Amaladoss, A., Haribhai, P. K. (2023). Hydroponics: current trends in sustainable crop production. Bioinformation, 19(9):925–938.
10. Fathidarehnijeh, E., Nadeem, M., Cheema, M., Thomas, R., Krishnapillai, M., and Lakshman Galagedara, L. (2024). Current perspective on nutrient solution management strategies to improve the nutrient and water use efficiency in hydroponic systems. Canadian Journal of Plant Science, 104(2): 88-102.
11. Shavrukov, Y., Genc, Y., Hayes, J. (2012). The Use of Hydroponics in Abiotic Stress Tolerance Research, Hydroponics - A Standard Methodology for Plant Biological Researches, Dr. Toshiki Asao (Ed.), ISBN: 978-953-51-0386-8, InTech
12. Polivanova, O. B., Bedarev, V. A. (2022). Hyperhydricity in Plant Tissue Culture. Plants (Basel). 11(23):3313.
13. Blidar, C. F., Ilea, C., Tripon, I. M. (2016). The use of filter paper bridges for initiating in vitro culture of *Viola wittrockiana* F1 Matrix 'Blue Blotch'. Romanian Biotechnological Letters, 22 (6): 12088-12096
14. Ascough, G. D., Fennell, C. W. (2024). The regulation of plant growth and development in liquid culture. South African Journal of Botany, 70(2): 181–190.
15. Zheleznichenko, T. V., Muraseva, D. S., Erst, A. S., Kuznetsov, A. A., Kulikovskiy, M. S., & Kostikova, V. A. (2023). The Influence of Solid and Liquid Systems In Vitro on the Growth and Biosynthetic Characteristics of Microshoot Culture of Spiraea betulifolia ssp. aemiliana. International Journal of Molecular Sciences, 24(3), 2362.
16. Trejo-Téllez, L. I., & Gómez-Merino, F. C. (2012). Nutrient solutions for hydroponic systems. In Toshiki Asao (edt): Hydroponics – A Standard Methodology for Plant Biological Researches (pp. 1–22). InTech.
17. Rouphael, Y., & Colla, G. (2005). Growth, yield, fruit quality and nutrient uptake of hydroponically cultivated zucchini squash as affected by irrigation system and growing season. Scientia Horticulturae, 105(2), 177–195.
18. Wheeler, G. (2023). Nutrient management in hydroponic systems: Achieving optimal plant growth. J Agric Sci Bot. 8(2):234.
19. Laevens, G. C. S., Dolson, W. C., Drapeau, M. M., Telhig, S., Ruffell, S. E., Rose, D. M., Glick, B. R., & Stegelmeier, A. A. (2024). The Good, the Bad, and the Fungus: Insights into the Relationship Between Plants, Fungi, and Oomycetes in Hydroponics. Biology, 13(12), 1014.
20. Bunyuth, Y., Mardy, S. (2024). Hydroponic systems: an overview of benefits, challenges, and future prospects. Indonesian Journal of Social Economics and Agricultural Policy, 1(1):10-18
21. Moon, K. B., Park, J. S., Park, Y. I., Song, I. J., Lee, H. J., Cho, H. S., Jeon, J. H., & Kim, H. S. (2019). Development of Systems for the Production of Plant-Derived Biopharmaceuticals. Plants (Basel, Switzerland), 9(1), 30.
22. Wada, T., Sudo, K. and Usami, T. (2023). Suppression of waterborne diseases using Bacillus subtilis var. natto in hydroponic cultures. Acta Hortic. 1378, 1-6
23. Stanghellini, M. E., & Rasmussen, S. L. (1994). Hydroponics: A solution for zoosporic pathogens. Plant Disease, 78(12), 1129–1138.
24. Maucieri, C., Nicoletto, C., Os, E.v., Anseeuw, D., Havermaet, R.V., Junge, R. (2019). Hydroponic Technologies. In: Goddek, S., Joyce, A., Kotzen, B., Burnell, G.M. (eds) Aquaponics Food Production Systems, pp 77-110. Springer, Cham.
25. Jensen, M. H. (1997). Hydroponics. HortScience, 32(6), 1018–1021.
26. Barrett, G. E., Alexander, P. D., Robinson, J. S., & Bragg, N. C. (2016). Achieving environmentally sustainable growing media for soilless plant cultivation systems – A review. Scientia Horticulturae, 212, 220–234.
27. Horsch, R. B., Fry, J. E., Hoffmann, N. L., Eichholtz, D., Rogers, S. G., & Fraley, R. T. (1985). A simple and general method for transferring genes into plants. Science, 227(4691), 1229–1231.
28. Nosaki, S., Hoshikawa, K., Ezura, H., & Miura, K. (2021). Transient protein expression systems in plants and their applications. Plant biotechnology (Tokyo, Japan), 38(3), 297–304.
29. Murashige, T. and Skoog, F. (1962). A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. Physiol Plant. 15(3): 473–497.

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