

Chapter 3

Protocols for Root Developmental Studies in White Lupin

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Abstract

White lupin (*Lupinus albus*) is a legume species that develops two remarkable root adaptations to nutrient limitation: nitrogen-fixing nodules and dense cluster roots specialized for phosphate acquisition. These traits, together with the species' agronomic relevance and strong developmental plasticity, make white lupin a powerful model for studying local and systemic regulation of root development under nutrient stress. This chapter provides a comprehensive collection of experimental procedures designed to investigate cluster root formation, nodulation, and associated physiological responses at both cellular and whole-plant levels. We describe optimized hydroponic culture systems that allow precise control of nitrogen and phosphate availability, together with reliable seed sterilization and germination procedures. We present a robust protocol for *Agrobacterium rhizogenes*–mediated hairy-root transformation to investigate gene function in root tissues. A controlled nodulation assay using *Bradyrhizobium lupini* enables reproducible symbiotic infection and nodule development. A grafting method is also provided to dissect local versus systemic contributions to root architectural responses. Finally, we detail three in situ physiological assays—phosphatase activity, ferric reductase activity, and proton efflux—that collectively allow the visualization of key functional traits associated with nutrient foraging. Together, these protocols provide an integrated toolkit for exploring the developmental and physiological mechanisms underpinning root plasticity and symbiotic regulation in white lupin.

Keywords White lupin, Cluster roots, Nodulation, Root plasticity, Hydroponic culture, Hairy-root transformation, *Bradyrhizobium lupini*, Grafting technique, Phosphate deficiency, Physiological assays

1 Introduction

The white lupin (*Lupinus albus*) is a Mediterranean legume valued for its high seed protein content and for its exceptional root developmental plasticity under nutrient limitation [1, 2]. In response to phosphate deficiency, white lupin forms dense cluster roots composed of numerous short third-order lateral roots specialized for the mobilization and acquisition of inorganic phosphate [3, 4]. Under nitrogen deficiency, it establishes a symbiotic interaction with *Bradyrhizobium lupini*, leading to the formation of nitrogen-fixing nodules. These two adaptive responses provide an experimentally tractable system

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to study nutrient sensing, systemic regulation, and coordinated root architectural changes.

White lupin is therefore an increasingly used model for investigating the molecular and physiological mechanisms underlying root plasticity, nutrient foraging, and symbiotic development. However, the successful implementation of experimental approaches in this species requires tightly controlled growth conditions, reproducible transformation methods, and quantitative assays that capture key physiological activities associated with nutrient uptake.

This chapter presents a set of complementary methods enabling the functional and physiological analysis of cluster root development and nodulation. These protocols are designed to be accessible, robust, and adaptable, allowing researchers to combine developmental, physiological, and molecular approaches within a unified experimental framework.

This chapter describes five major procedures:

(1) hydroponic culture systems enabling precise control of nitrogen and phosphate supply;

(2) *Agrobacterium rhizogenes*–mediated hairy-root transformation for gene functional analysis;

(3) controlled nodulation assays using *B. lupini*;

(4) a stem grafting method to dissect local versus systemic regulatory pathways; and

(5) three in situ physiological assays quantifying phosphatase activity, ferric reductase activity, and proton efflux.

2 Materials

2.1 Plant Material

- White lupin plants: *Lupinus albus* cv. AMIGA (Florimond Desprez, France).

2.2 Bacteria Strain

1. *Bradyrhizobium lupini* MIAE428 (MIAE collection, AgroEcologie INRAE Dijon) (formerly LL13).
2. *Agrobacterium rhizogenes* ARqua1.

2.3 White Lupin Hydroponic Culture (see Note 1)

1. Phosphate-deficient nutrient solution: 400 µM $Ca(NO_3)_2$, 54 µM $MgSO_4$, 0.24 µM $MnSO_4$, 0.1 µM $ZnSO_4$, 0.018 µM $CuSO_4$, 2.4 µM H_3BO_3, 0.03 µM Na_2MoO_4, 10 µM Fe–EDTA, 200 µM K_2SO_4. Store at 4°C.
2. Phosphate-rich nutrient solution: 400 µM $Ca(NO_3)_2$, 54 µM $MgSO_4$, 0.24 µM $MnSO_4$, 0.1 µM $ZnSO_4$, 0.018 µM $CuSO_4$, 2.4 µM H_3BO_3, 0.03 µM Na_2MoO_4, 10 µM Fe–EDTA, 400 µM KH_2PO_4. Store at 4°C.

3. Nitrogen-free nutrient solution: 54 µM $MgSO_4$, 0.24 µM $MnSO_4$, 0.1 µM $ZnSO_4$, 0.018 µM $CuSO_4$, 2.4 µM H_3BO_3, 0.03 µM Na_2MoO_4, 10 µM Fe–EDTA, 400 µM KH_2PO_4. Store at 4°C.
4. Growth conditions: 16 h light/8 h dark, 25°C day/20°C night, 65% relative humidity, photon flux density 200 µmol/m^2/s.
5. 3-L pot or 200-L tank.
6. Air pump to ensure nutrient solution aeration.

2.4 Seed Sterilization and Germination on Plate

1. Sterilization solution: 20% solution prepared using Halonet (Proquimia, 7% sodium hypochlorite, 1% sodium hydroxide) and water.
2. 0.5× MS solid medium: 2.2 g/L Murashige and Skoog Basal medium, 10 g/L Saccharose, 0.5 g/L MES, 7 g/L Agar, pH 5.7.
3. Square Petri dishes (12 × 12 cm).
4. Sterile Milli-Q water.

2.5 Nodulation Assay

1. Substrate: 1:1 (v/v) mixture of zeolite 0.7–3 mm and zeolite 0.5–1.0 mm.
2. YM medium: 10 g/L Mannitol, 1 g/L yeast extract, 0.5 g/L K_2HPO_4, 50 mg/L NaCl, 100 mg/L $MgSO_4 \cdot 7H_2O$, 40 mg/L $CaCl_2$, 0.43 g/L glutamic acid, 4 mg/L $FeCl_3$. Sterilize using an autoclave.
3. Nalidixic acid stock: 20 mg/mL solution in sterile water.
4. Containers: Magenta GA-7 pots 77 × 77 × 97 mm (standard and perforated).
5. Cotton cord.

2.6 Hairy-Root Transformation

1. LB liquid and solid medium. 10 g/L Tryptone, 5 g/L Yeast extract, 5 g/L NaCl, add 15 g/L Agar for solid medium.
2. LB Glucose 1% solid medium. 10 g/L Tryptone, 5 g/L Yeast extract, 5 g/L NaCl, 15 g/L Agar, 10 g/L of D+ Glucose.
3. pK7m24GW-CR vector: pK7m24GW with constitutive pUbi:*mCherry*-ER:t35s cassette allowing identification of transformed roots by red fluorescence (*see* **Note 2**).
4. Antibiotics stock solution:
   Kanamycin 30 mg/mL solution in sterile water.
   Streptomycin 20 mg/mL solution in sterile water.
   Spectinomycin 100 mg/mL solution in sterile water.
5. Acetosyringone.

6. Tools and substrate: Scalpel blade; vermiculite; mini-greenhouse lid.
7. Hydroponic pots and aeration system.
8. FastGene Blue/Green LED Flashlight.

2.7 Physiological Activity Assay

2.7.1 Phosphatase Activity Assay

1. 5-Bromo-4-chloro-3-indolyl phosphate (BCIP) stock solution: 65 mg of BCIP dissolved in 5 mL of DMSO.
2. Phosphatase activity gel: 1.2 g of agar in 150 mL of 50 mM sodium acetate buffer (pH 5), supplemented with 1.5 mL of BCIP solution. Pour onto a 24 × 24 cm square Petri dish lid and allow to solidify (*see* **Note 3**).
3. Plastic wrap.

2.7.2 Ferric Reductase Activity Assay

1. Reagents: 100 mM Ferric Sodium EDTA (FeNaEDTA) solution; bathophenanthroline disulfonic acid disodium salt (BPDS).
2. Ferric reductase gel: 1.2 g of agar, 26 mg of K_2SO_4 in 150 mL of distilled water, supplemented with 150 µL of 100 mM FeNaEDTA and 24 mg of BPDS. Pour onto a 24 × 24 cm square Petri dish lid and allow to solidify (*see* **Notes 3–5**).
3. Plastic wrap.

2.7.3 Proton Exudation Assay

1. Bromocresol Purple solution (BCP) stock solution: 25 mg of BCP dissolved in 5 mL of 500 mM Tris-HCl buffer (pH 6).
2. Proton exudation gel: Dissolve 1.2 g of agar, 26 mg of $CaSO_4 \cdot 2\,H_2O$, 52 mg of K_2SO_4 in 150 mL of distilled water. Autoclave, let it cool, and add 1.5 mL of 100× BCP solution. Pour onto a 24 × 24 cm square Petri dish lid and allow to solidify (*see* **Note 3**).
3. Plastic wrap.

3 Methods

3.1 Seed Sterilization and Germination

1. Dispense up to 35 seeds into a 50 mL tube and add 20% Halonet solution. Incubate for 20 min on slow agitation (*see* **Note 6**).
2. Remove the sterilization solution and wash seeds 5–6 times with sterile Milli-Q water until no bubbles remain.
3. Immerse the seeds in sterile Milli-Q water for 2 h in a square Petri dish (*see* **Note 7**).

4. Remove the water and allow seeds to dry for 10 min in the laminar flow.
5. Place seeds on 0.5× MS solid medium in square Petri dishes with the hilum facing downward. Press seeds gently into the agar to ensure proper imbibition and prevent displacement.
6. Seal the plates with parafilm and cover them with paper to protect from light.
7. Incubate plates at an angle of 45° in a growth chamber for 3 days.

3.2 Hairy-Root Transformation

Hairy root transformation in *L. albus* is performed using *A. rhizogenes* strain ARqua1, generating composite plants with transformed roots and unaltered shoots. Transformed roots exhibit a characteristic hairy-root phenotype alongside non-transformed, unaltered roots. We used the pK7m24GW-CR vector, a Gateway-compatible destination vector, which was modified by introducing a constitutive pUbi:*mCherry*-ER:t35s cassette [5] *see* **Note 2**) into the pK7m24GW [6] vector (Fig. 1A), allowing identification of transformed roots by red fluorescence.

3.2.1 Preparation of Agrobacterium rhizogenes ARqua1

1. Streak *A. rhizogenes* ARqua1 from a glycerol stock onto LB agar containing 20 µg/mL streptomycin (bacterial selection) and 100 µg/mL spectinomycin (vector selection) if using a

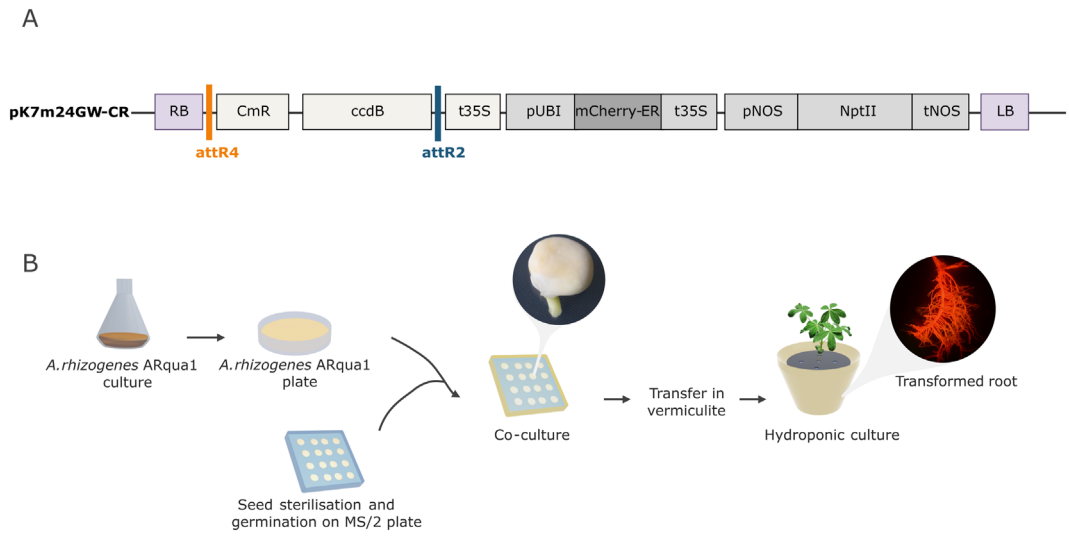


Fig. 1 Plasmid map of the Gateway backbone pK7m24GW-CR used for hairy-root transformation (**A**). Schematic overview of the major steps involved in *Lupinus albus* hairy-root transformation (**B**), including *Agrobacterium rhizogenes* culture, seed sterilization and germination, cocultivation with an image of a germinated seed with the primary root cut, transfer to vermiculite, and subsequent hydroponic culture. The image shows a transformed hairy root of *L. albus*. Schematics were designed using Blender software

pK7m24GW-CR Gateway-based vector. Incubate at 28°C for 3 days.

2. Inoculate a single colony into 2 mL of LB liquid medium with antibiotics and incubate in a shaker at 28°C, 150 rpm for 24 h.
3. The day before transformation, spread 200 µL of the overnight culture onto LB + 1% Glucose plates supplemented with 100 µM of acetosyringone and antibiotics. Incubate overnight at 28°C (*see* **Note 8**).

3.2.2 Transformation and Coculture (Fig. 1B)

1. Sterilize and germinate seeds as described in Sect. 3.1. Incubate seedlings for 43–45 h until primary roots reach 1–1.5 cm (*see* **Note 9**).
2. Cut each root at mid-length.
3. Gently scrape the cut root surface across the *A. rhizogenes* ARqua1lawn on the LB + 1% glucose, 100 µM acetosyringone plate prepared the day before transformation (*see* **Note 10**).
4. Transfer each seedling onto 0.5× MS solid medium containing 25 µg/mL kanamycin, positioning the cut root surface in contact with the medium to prevent adventitious rooting (*see* **Note 11**).
5. Incubate plates at a 20° angle under long-day conditions (16 h light/8 h dark, 20°C).
6. After 6 days of coculture, transfer seedlings to moist vermiculite (*see* **Note 12**). Remove the seed coat when possible and place seedlings with the cut root surface facing downward. Ensure cotyledons rest on the vermiculite surface to prevent desiccation.
7. Cover with a mini-greenhouse lid for 10 days to aid acclimation (*see* **Note 13**).

3.2.3 Transfer to Hydroponics

1. Prepare hydroponic 3-L pots with nutrient solution and aeration.
2. Remove transformed seedlings from vermiculite after 10 days and rinse roots gently with water (*see* **Note 14**).
3. Transfer plants to hydroponic pots containing the appropriate nutrient solution.
4. Replace nutrient solution weekly (*see* **Note 15**).
5. For phosphate deficiency experiments, harvest cluster roots after 20–30 days of hydroponic culture (*see* **Notes 16 and 17**).
6. Chimeric roots containing non-transformed regions may be obtained. When using plasmid construction from pK7m24GW-CR, the transformation status of the analyzed root region should be verified by the presence of red fluorescence excited by a handheld FastGene Blue/Green LED flashlight.

3.3 Nodulation Assay

3.3.1 Bradyrhizobium Lupini MIAE428 Culture

1. Three days before inoculation, inoculate *B. lupini* MIAE428 into 5 mL of YM medium containing 20 µg/mL nalidixic acid
2. Incubate at 28°C, 150 rpm for 3 days until OD_{600} reaches the appropriate value (*see* **Note 18**).

3.3.2 Plant Inoculation and Nodulation

1. Prepare a 1:1 (v/v) mixture of zeolite 0.7–3 mm and zeolite 0.5–1.0 mm.
2. Wash the substrate with Milli-Q water until rinse water runs clear (*see* **Note 19**).
3. Thread a cotton wick through the perforated Magenta GA-7 pot so that it reaches the bottom of the second, non-perforated pot.
4. Fill the upper pot completely with the substrate mixture, ensuring the cotton wick is fully embedded. Pack down well.
5. Cover with two sheets of aluminum foil and sterilize the entire assembly (*see* **Note 20**).
6. Sterilize and dispense seeds on solid 0.5× MS medium as described in Sect. 3.1 and incubate the plates at an angle of 45°C in a growth chamber.
7. Transfer 3-day-old seedlings to the sterilized pots and keep the foil cover to maintain humidity (*see* **Note 9**).
8. Inoculate 7-day-old seedlings with 500 µL of *B. lupini* culture applied close to the root system.
9. Refill the above tank with medium as soon as necessary.
10. Observe nodules 3–4 weeks after inoculation (Fig. 2).

3.4 V-Shape Grafting

1. Germinate seeds for 4 days in vermiculite.
2. Transfer seedlings to aerated hydroponic nutrient solution (*see* **Note 14**).
3. At day 6, cut the stem horizontally to separate the scion and rootstock using a scalpel with a new blade.
4. Cut the top of the rootstock vertically and prepare the scion with a thin bevel (V-shape graft) (Fig. 3).
5. Insert the scion bevel into the slit of the rootstock and wrap tightly with parafilm to avoid any gaps (*see* **Note 21**; Fig. 3).
6. Place grafted seedlings in a large (200 L) tank of nutrient solution (*see* **Notes 22 and 23**).
7. Cover plants with a greenhouse dome for 4 days to maintain high humidity.
8. Over the next 21 days, inspect grafts regularly. Remove root growth emerging through the parafilm and rewrap with fresh parafilm as needed (*see* **Note 24**).

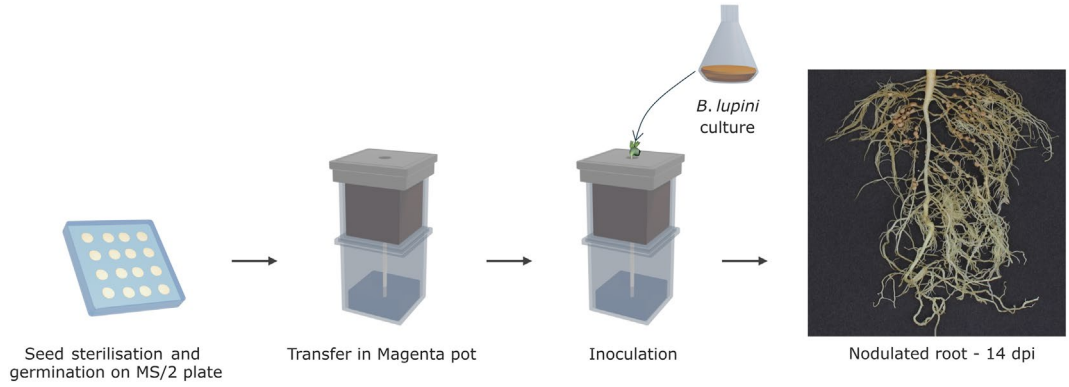


Fig. 2 Overview of the major steps of the nodulation assay in *Lupinus albus* with *Bradyrhizobium lupini*: Seeds were sterilized and germinated, then transferred to Magenta GA-7 pots containing a zeolite mixture and inoculated with the symbiotic bacterium *B. lupini* MIAE428. Nodules can be visible at 14 days post inoculation (dpi), as shown in the photograph of a lupine root grown under nitrogen-deficient conditions at 14 dpi. Schematics were designed using Blender software



Fig. 3 V-shaped grafting of lupin seedlings: the first panel shows the horizontal cut of the rootstock and the V-shaped bevel prepared on the scion. The second panel shows the scion inserted into the rootstock, and the final panel shows the assembled graft, tight with parafilm

3.5 Physiological Activity Assay

3.5.1 Phosphatase Activity Assay

1. Rinse the root system with Milli-Q water and spread it flat onto the phosphatase activity gel.
2. Cover roots with plastic wrap, removing air bubbles (*see* **Notes 25 and 26**).
3. Incubate for the required time until blue signal develops (*see* **Notes 27–30**; Fig. 4A).

3.5.2 Ferric Reductase Activity Assay

1. Rinse the root system with Milli-Q water and spread it flat onto the ferric reductase gel.
2. Cover with plastic wrap and remove air bubbles (*see* **Notes 25 and 26**).
3. Protect from light and incubate 4–5 h until a red/pink coloration develops (*see* Notes **28–30**; Fig. 4B).

3.5.3 Proton Exudation Assay

1. Rinse the root system with Milli-Q water and spread it flat onto the proton exudation gel.
2. Cover with plastic wrap and remove air bubbles (*see* **Note 26**).
3. Protect from light and incubate 4–5 h until purple-to-yellow color changes reveal acidification zones (*see* **Notes 28–31**; Fig. 4C).

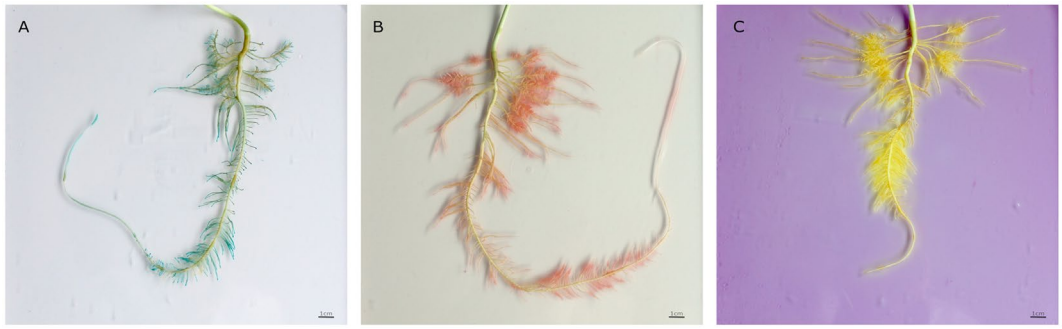


Fig. 4 Physiological activity assays. Phosphatase activity: image of a plant root system grown in a phosphate-rich, iron-free nutrient solution and after 4 h of incubation on gel (**A**); ferric reductase activity assay: image of a plant root system grown under phosphate-deficient conditions and after 2 h of incubation on gel (**B**); proton exudation assay: Image of a plant root system grown in phosphate-deficient conditions and after 4.5 h of incubation on gel (**C**)

4 Notes

1. Adequate aeration using an air pump in the growth medium is a critical parameter. In addition, culture volume affects lupin root system architecture: the developmental kinetics of cluster roots differ between plants grown in 3-L pots and those grown in 200-L tanks.
2. Briefly, the pUbi:*mCherry*-ER:t35s cassette from Fournier et al. 2018 [5] was PCR-amplified using specifically designed primers (5′-CGGCACTAGTCTAAAACCAAAATC-3′ and 5′-GCGCACTAGTAGGTCACTGGATTT-3′), each containing a *SpeI* restriction site. The resulting PCR product was ligated into the pk7m24GW vector, which had been previously digested with *XbaI* enzyme.
3. When preparing agar gels, repeatedly stop the microwave and swirl gently to avoid local overheating. Overheating can caramelize sugars in buffer components, introducing color backgrounds that interfere with colorimetric detection.
4. The ferric reductase assay must always be carried out in the dark. Even brief exposure to light during incubation will affect BPDS chemistry and generate spurious red signals.
5. FeNaEDTA solutions should be prepared fresh whenever possible. Aged solutions show partial iron oxidation and diminish the sensitivity of the ferric reductase assay.
6. Seeds that turn strongly yellow or orange during sterilization should be discarded. They generally reflect compromised seed coats or internal damage and germinate poorly.
7. Ensure seeds remain fully immersed during the 2 h water imbibition step. Uneven hydration leads to heterogeneous germination and variable root lengths at the time of transformation.
8. LB Glucose 1% plates supplemented with acetosyringone should be prepared on the same day as the bacterial streaking step. Acetosyringone degrades rapidly, reducing the induction of virulence genes and transformation efficiency.
9. For hairy-root transformation, seedling age and root length uniformity are critical. Transforming seedlings with roots >1.5 cm drastically reduces the frequency of transformed roots and increases callus formation.
10. When scraping seedlings on *A. rhizogenes*, avoid collecting excessive bacterial mass. Overloading the wound increases contamination risks and reduces plant survival. A thin, even smear of bacteria is sufficient.

11. Ensure the cut root end of the seedling remains in tight contact with kanamycin-containing medium during coculture. Poor contact allows escape of wild-type adventitious roots, reducing the proportion of transgenic roots.
12. Removing the seed coat before transplanting to vermiculite improves survival but should be done carefully. Removing it too early or too aggressively can detach cotyledons, which severely reduces plant vigor.
13. Maintaining stable humidity under the mini-greenhouse lid is essential during the first 10 days post-transformation. Drying episodes commonly result in the collapse of transformed roots or the cessation of growth.
14. For hydroponic transfer, rinse vermiculite completely to avoid the transfer of fine particles that may accumulate around roots and create local hypoxic zones in the nutrient solution.
15. Weekly replacement of the nutrient solution is essential for consistent phosphorus or nitrogen treatments. White lupin roots modify their rhizosphere chemistry intensely; old solutions quickly become chemically heterogeneous.
16. When analyzing cluster root responses, ensure plants are grown at comparable developmental stages. Cluster root induction is highly stage-dependent and can vary several-fold between plants differing by only a few days.
17. For long experiments (20–30 days of hydroponics), algae growth can occur in the nutrient solution. Use opaque covers or frequent cleaning to avoid light penetration, as algal biofilms alter nutrient availability and local pH. This problem is much less prevalent in phosphate deficiency conditions.
18. *Bradyrhizobium lupini* grows slowly. Ensure cultures are incubated for a full 72 h for optimal OD_{600} values. Under-grown cultures reduce nodulation efficiency, while over-grown cultures may lead to clumping and uneven inoculation.
19. Rinse the zeolite mix substrate thoroughly until the wash water runs completely clear. Residual dust significantly reduces aeration and may retain inhibitory ions, interfering with early nodulation.
20. During the first days after transfer into the Magenta pots, maintaining high humidity with aluminum foil is essential. Seedlings dessicate rapidly when the substrate dries even slightly.
21. During grafting, the scion bevel must match the width of the rootstock slit precisely. Any lateral gap greatly promotes oxidative events, resulting in graft failure within 48 h.

22. Grafting is very difficult to achieve in phosphate-deficient conditions.
23. After transferring grafted plants to hydroponics, slight movement of the nutrient solution can disturb fragile graft unions. Reduce aeration strength during the first days until graft healing stabilizes.
24. When rewrapping parafilm around graft junctions, avoid constricting the stem. Restrictive wrapping inhibits vascular reconnection and may lead to swelling or cracking of the junction.
25. Roots must be fully flattened on the gels for all physiological assays. Air pockets between root tissue and agar lead to incomplete contact and false-negative zones in the reaction patterns.
26. When spreading roots for physiological assays, avoid mechanical damage. Damaged tissues can release oxidative compounds that react nonspecifically with BCIP or BPDS, causing artifacts.
27. Phosphatase activity development time varies with plant age and phosphate status. Strongly induced cluster roots may develop color within 30–60 min, whereas control roots may require several hours. Avoid over-incubation, which can cause background staining.
28. For physiological assays involving color reactions, always include a negative control root (e.g., from nutrient-replete conditions) to validate specificity. Background coloration occasionally develops in gels stored too long.
29. When imaging roots after assays, keep gels hydrated and avoid direct exposure to strong lights. Both BCIP and bromocresol purple signals can fade under intense illumination.
30. If roots detach from gels before imaging, gently reposition them with forceps under plastic wrap. Reexposure to air will dry roots rapidly and distort signals.
31. Proton exudation patterns depend on both genotype and nutrient history. Roots previously exposed to high-phosphate conditions tend to acidify more slowly than those grown under phosphate deficiency. Interpret timings accordingly.

Competing Interests

The authors have no conflicts of interest to declare that are relevant to the content of this chapter.

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