Legume Science

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ORIGINAL ARTICLE OPEN ACCESS

An Optimised Protocol for Faba Bean (*Vicia faba* L.) Tissue Culture and Transformation

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Received: 23 May 2025 | **Revised:** 27 October 2025 | **Accepted:** 8 December 2025

Keywords: faba bean | GFP | particle bombardment | root induction | tissue culture | transformation

ABSTRACT

Faba bean (*Vicia faba* L.) is an important legume that contributes to agricultural sustainability through nitrogen fixation and offers substantial nutritional benefits. Nonetheless, its genetic enhancement has been constrained by challenges associated with tissue culture and transformation. This study presents the development and optimisation of a robust and reproducible protocol for tissue culture and genetic transformation in various *V. faba* genotypes. Thirty-three genotypes were evaluated with various hormone combinations to enhance shoot and root regeneration. Among these, 1.5 mg/L indole-3-acetic acid (IAA) demonstrated the highest efficacy for uniform root formation across all genotypes. Genetic transformation was performed through particle bombardment, utilising excised embryos as the target tissues. A transformation efficiency of 11.7% was achieved in cultivar Tiffany, demonstrating stable transgene inheritance. This protocol mitigates significant limitations in faba bean regeneration, such as the production of phenolic compounds and variability in rooting, thereby facilitating consistent transformation across cultivars. This study presents an efficient and reproducible protocol for tissue culture and transformation in *V. faba* genotypes. The approach establishes a practical foundation for subsequent applications in gene editing and molecular breeding of faba bean and a potential methodological basis for optimisation in other legume crops.

1 | Introduction

Faba bean (*Vicia faba* L.) is a legume crop with considerable nutritional and economic significance for sustainable food production (Bangar and Kajla 2022; De Cillis et al. 2019). Due to the rich seed content of protein and other nutrients, it is an important source of food for both humans and animals (Chaudhary et al. 2022). Furthermore, faba bean is known for its ability to fix atmospheric nitrogen and generate a post-harvest soil-nitrogen surplus, reducing fertiliser requirements and enriching soils in crop rotations (Youseif et al. 2017). However, the full potential of faba bean production is constrained by diverse factors including biotic and abiotic stress and low yield stability (De Cillis et al. 2019; López-Bellido et al. 2005; Lyu et al. 2021), and breeding is hindered by a low seed multiplication rate and a strong reliance on insect pollinators. As a result, breeding progress for faba bean is currently low and cultivar development can take up to 15 years from an initial cross until cultivar registration and release. Considering these constraints and difficulties, breeding progress in faba bean could profit enormously from new breeding technologies like CRISPR-mediated gene editing. However, *V. faba* is rather recalcitrant to tissue culture regeneration, meaning that genetic engineering and gene editing methods have only rarely been successfully applied in faba bean research and breeding to date.

The utilisation of tissue culture, transformation and gene editing techniques facilitates modifications in plants and their developmental processes, potentially fostering agricultural progress through accelerated crop genetic improvement. Earlier

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studies on faba bean transformation demonstrated the use of *Agrobacterium*-mediated transformation for the incorporation of the β-glucuronidase (GUS) reporter gene (Schiemann and Eisenreich 1989). Subsequently, transgenic *V. faba* transformants were generated from *Agrobacterium*-infiltrated stem segments (Böttinger et al. 2001) and embryo axes (Hanafy et al. 2005). Anwar et al. (2011) outlined an in vitro regeneration protocol for faba bean; however, the transformation and regeneration of *V. faba* continue to present significant challenges, characterised by low transformation frequencies and considerable time requirements (Hanafy et al. 2005). Previous studies have indicated comparable challenges in developing effective transformation and regeneration systems for other leguminous plants, which are generally recalcitrant for tissue culture regeneration and transformation (Bean et al. 1997).

Previous transformation efforts in faba bean primarily utilised *Agrobacterium*-mediated methods, whereas our study employs biolistic particle bombardment, which theoretically enables the genetic transformation of any genotype within a species (Sanford et al. 1993). However, effective transformation in faba bean has been challenging due to multiple factors, including explant source, culture media formulations, growth regulators and transformation methods (Gantait and Mukherjee 2021; Hanafy et al. 2005). In addition, faba bean varieties intended for human consumption must meet specific agronomic and nutritional criteria. By establishing an efficient and reliable protocol for both the reference genome-sequenced accession Hedin/2 and the present-day German faba bean cultivar Tiffany (NPZ Lembke KG, Hohenlieth, Germany), which fulfils these desirable traits (German Federal Variety Registration list 2024). This study provides a strong foundation for advancing both tissue culture and genetic transformation efforts in the legume species. An integral part of this protocol is the optimised tissue culture system that enables consistent shoot regeneration and robust root formation across diverse genotypes.

2 | Materials and Methods

2.1 | Plant Materials

A total of 33 genotypes were used in this study, including 19 diverse cultivars from Germany, Canada and the United Kingdom along with 14 proprietary breeding lines obtained from Norddeutsche Pflanzenzucht Hans-Georg Lembke KG (Holtsee, Germany). Alongside Hedin/2 and Tiffany, from which high-quality annotated genome assemblies are available (Jayakodi et al. 2023), the panel includes several recently released cultivars from these three countries (Table 1), making the study particularly relevant for modern breeding efforts. Mature seeds and fresh embryos were used as the source material for the tissue culture experiments.

TABLE 1 | List of cultivars and breeding lines used in tissue culture experiments.

Cultivar or line name	Country of origin	Year of release
FB-0050	Germany	n.a.
FB-0206	Germany	n.a.
FB-0073	Germany	n.a.
FB-0110	Germany	n.a.
FB-0052	Germany	n.a.
Alfred	Germany	1993
Allison	Germany	2019
Bolivia	Germany	2021
DL Nevado	Canada	2024
Dosis	Germany	2021
Expresso	Germany	2003
FB-0130	Germany	n.a.
FB-0132	Germany	n.a.
Hedin/2	Germany	1986
Hiverna-Sublinie	Germany	1986
Kristall	Germany	1973
Loki	Germany	2025
FB-0134	Germany	n.a.
Lynx	UK	2018
Macho	Germany	2018
Mallory	Canada	1993
FB-0121	Germany	n.a.
Pantani	UK	1995
FB-0131	Germany	n.a.
Scirocco	Germany	1992
FB-0136	Germany	n.a.
Tiffany	Germany	2015
Trumpet	Germany	2015
FB-0129	Germany	n.a.
Victus	Canada	2020
FB-0137	Germany	n.a.
Yukon	Canada	1998
FB-0125	Germany	n.a.

2.2 | Evaluation of the Explant and Standardisation of the Media

2.2.1 | Callus Generation From Seeds

Fifteen seeds from each variety were utilised, ensuring they were free from damage and infection. Seeds were surface sterilised using 70% ethanol (5 min) followed by 3% sodium hypochlorite (30 min), rinsed three to four times with sterile Milli-Q water and soaked overnight. Cotyledons were excised carefully to avoid damage. Subsequently, the seeds were transferred to Murashige and Skoog medium (MS) (Murashige and Skoog 1962) (Table S6) containing different hormone concentrations for callus induction

(Table S1). The media for callus induction contained the auxins 2,4-dichlorophenoxyacetic acid (2,4-D) and naphthaleneacetic acid (NAA), essential for cell differentiation, promoting the formation of embryogenic calli necessary for subsequent regeneration steps. Halved cotyledons were gently pressed against the medium with the flat side of the endosperm facing downwards and incubated in darkness at 23°C ± 2°C with 40%–60% humidity. Subculturing was carried out every 10–12 days to promote embryogenic callus induction and reduce phenolic accumulation.

2.2.2 | Shoot Generation From Callus

Friable embryogenic calli derived from seeds were transferred to MS medium (Table S6) supplemented with various hormones (MS + 09, MS + 11, MS + 12), as specified in Table S1. Cultures were maintained under 16 h light/8 h dark at 23°C ± 2°C and subcultured every 10–12 days to maintain tissue viability and reduce browning. To promote shoot elongation, the callus were placed in MS + 12 (Table S1). After transferring calli from dark culture conditions to shoot induction media (MS + 12), shoot regeneration from callus was observed within approximately 2 weeks under light conditions.

2.3 | Embryo Extraction and Plant Regeneration

For the regeneration of plants from embryos, 10–15 seeds from each cultivar were sterilised as previously described, and sterilised seeds were placed overnight in darkness on MS medium with sterile Milli-Q water. Subsequently, aseptic excised embryos were placed on MS medium supplemented with specific hormones (MS1) (Table S4). MS1 medium, containing 6-benzylaminopurine (BAP) and kinetin (KIN) with low NAA, stimulated shoot initiation and elongation, with regeneration visible after approximately 2 weeks. Plants were maintained under standard growth conditions as mentioned above.

2.4 | Optimisation of Root Formation Using Hormone Treatment

Various hormone treatments can be implemented to enhance root development. The hormones NAA, indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA), were tested at four concentrations (0.1, 0.5, 1.0 and 1.5 mg/L). Initially, cultivar Tiffany was used for optimisation. The seeds were soaked overnight in sterilised Milli-Q water, followed by aseptic embryo excision. The embryos were initially cultured on MS1 medium (Table S4) for 10 days. Subsequently, the embryos were transferred to MS2 medium supplemented with one of the respective hormones NAA, IAA and IBA at four different concentrations (0.1, 0.5, 1.0 and 1.5 mg/L). MS2 medium (Table S7), characterised by reduced nutrient and sucrose levels (half-strength MS), was utilised to gradually decrease osmotic stress, stabilising embryogenic tissues and preparing them for efficient organogenesis in subsequent regeneration stages. The cultures were maintained under controlled conditions with a photoperiod of 16-h light/8-h dark at 23°C ± 2°C. Each treatment was repeated three times. After determining the optimal hormone concentration with cultivar Tiffany, this refined concentration was employed to assess shoot and root development in 32 additional genotypes. Embryos from all genotypes were subjected to uniform culture conditions, and their responses to the optimised hormonal treatment were monitored. Plants with established roots and shoots were transferred to pots filled with vermiculite–soil mixture and transferred to greenhouse under controlled conditions.

2.5 | Genetic Transformation by Particle Bombardment Method

The transformation experiment used two cultivars, Tiffany and Hedin/2. The seeds were soaked overnight in sterilised Milli-Q water, after which the embryos were aseptically excised. Excised sterilised embryos of these two cultivars were maintained on MS osmotic (MSO) medium (MS + B5 vitamins: 4.4 g/L, sucrose: 30 g/L, sorbitol: 50 g/L, mannitol: 50 g/L and agar: 7 g/L with pH: 5.8) for 2 h. The transformation was carried out using the pLH6000-GFP construct, which contained *hygromycin phosphotransferase* (*hpt*) with the CaMV35S promoter and the nopaline synthase (nos) terminator and *green fluorescent protein (gfp)* under the maize ubiquitin promoter with the *Agrobacterium tumefaciens* nos terminator (Imani and Kogel 2020; Zhuo and Yang 2004). The bombardment was conducted using the protocol from Mookkan (2018), and transgenic plants were generated from bombarded embryos. Confocal microscopy was used to observe *green fluorescent protein* (*gfp*) expression in embryos 48 h after the bombardment. The excitation wavelength was set to 488 nm, and fluorescence emission was detected in the range of 500–550 nm.

2.6 | Regeneration and Confirmation of the Transgenic Plants

Subsequent to particle bombardment, the embryos were first cultured on MS1 medium (Table S4) for a duration of 10 days to promote initial recovery and shoot regeneration. For the selection of putative transgenic events, hygromycin (50 mg/L) was applied 10 days after transformation. However, to improve rooting efficiency and minimise stress, hygromycin selection was omitted during the rooting stage. Root formation medium (MS3) (Table S5) included auxin (IAA) to specifically induce robust root formation, preparing regenerated plants for successful soil acclimatisation. Following regeneration, the plants were transferred to pots containing a mixture of 50% soil and 50% vermiculite and subsequently placed in greenhouse under controlled conditions. Leaf samples at the three to four leaf stage were collected, and genomic DNA was extracted using the method outlined by Doyle and Doyle (1990). The DNA was quantified using the Qubit broad-spectrum kit. Transformation of plants was verified through PCR analysis of the *hpt* marker gene. The detailed protocol for PCR amplification is provided in Table S3.

2.7 | Analysis of Transgene Inheritance

To evaluate transgene inheritance, transformation was conducted using 60 embryos of the cultivar Tiffany. T0 plants were selected based on PCR amplification of the *hpt* gene, transferred to pots containing a 1:1 mixture of soil and vermiculite and maintained in the greenhouse (21°C ± 2°C, 40%–60% relative

humidity). Seeds were collected from the T0 plants. For T1 screening, five to six seeds from selected T0 plants were germinated on MS2 medium (Table S7) under culture room conditions (16-h light/8-h dark, 23°C ± 2°C, 40%–60% humidity), then transferred into soil–vermiculite mix and grown in the greenhouse under the same conditions. Genomic DNA was extracted from T1 leaf tissue using the method described by Doyle and Doyle (1990). PCR amplification was performed using primers specific to the *hpt* gene to confirm transgene presence. PCR products were visualised on a 1% agarose gel to verify stable integration and inheritance of the transgene. The primer sequences were:

- *hpt*-F: 5′-GAACTCACCGCGACGTCTGTCGAG-3′
- *hpt*-R: 5′-GAAGTAGCGCGTCTGCTGCTCCATA-3′

2.8 | Statistical Analysis

Rooting response data were analysed using a two-way analysis of variance (ANOVA) in R (version 4.4.3) (R Core Team 2025) to assess the effects of hormone type (NAA, IBA and IAA) and concentration (0.1, 0.5, 1.0 and 1.5 mg/L) on root formation. Each treatment was replicated three times, and in each replicate, five plants were evaluated for rooting response. The response variable was the number of plants that formed roots (out of five). The significance of the main and interaction effects was determined using ANOVA, and differences were considered statistically significant at $p \leq 0.05$.

3 | Results

3.1 | An Optimised Media Composition for Regeneration

3.1.1 | Callus Induction

Genotypic variation was observed during callus induction, as cultivars Alexia and Fuego and breeding lines FB-0073, FB-0110 and Yukon responded differently to the tested hormone combinations. An optimised tissue culture protocol was successfully established for efficient regeneration across these genotypes. Initial experiments employed seeds for callus induction, assessing six different media compositions (MS + 02 to MS + 06) on cultivars Alexia and Fuego. Upon exposure to light, the seeds exhibited direct regeneration instead of callus induction (Figure 1a,b). However, we observed considerable production of phenolic compounds in these cultivars after 12–14 days in the identical medium. After transferring the seeds to dark conditions, callus induction occurred in cultivar Fuego, with MS + 02, MS + 04 and MS + 06 (Figure 1c). However, breeding lines FB-0073, FB-0110 and cultivar Yukon generated white and friable embryogenic calli in three distinct media compositions under dark conditions (Figure 1d–f). These calli exhibited clear morphological differences among the three media, where MS + 02 produced compact brownish calli, MS + 04 yielded soft light-yellow calli and MS + 06 generated friable white embryogenic calli with higher proliferation potential (Table S2). The hormone combination (MS + 09) facilitated embryogenic callus induction in line FB-0110 and FB-0073 with significant efficiency.

3.1.2 | Shoot Regeneration

Subsequently, the callus was transferred to culture media (MS + 11 to MS + 13) (Table S1) to evaluate their potential for shoot development, and genotype FB-0073 exhibited successful regeneration (Figure 1g–i). Among these, MS + 11 and MS + 12 were more effective in promoting the formation of compact embryogenic calli in genotype FB-0073, which served as precursors for regeneration. The addition of KIN in MS + 12 appeared to enhance cytokinin synergy, promoting finer, more friable embryogenic tissue in FB-0073 compared with MS + 11. In contrast, MS + 13 favoured shoot elongation and greening rather than embryogenic callus formation in FB-0073, likely due to its higher cytokinin-to-auxin ratio (Table S1). After utilising the callus for transformation, we encountered several challenges, including elevated phenolic compounds and insufficient shoot regeneration. Consequently, embryos were used as explants for all subsequent experiments. Using cultivar Tiffany, we used an MS1 media formulation (Table S4), which facilitated robust shoot regeneration from embryos. Tiffany exhibited vigorous and consistent shoot regeneration efficiency in this optimised MS1 medium. However, despite robust shoot growth, root formation was inadequate (Figure 2). This outcome demonstrated that although MS1 facilitated shoot regeneration, it failed to encourage root development, highlighting a limitation.

3.2 | Analysis of the Effects of Various Hormones on Root Regeneration

The effects of different plant growth hormones on root development were assessed using NAA, IBA and IAA at four concentrations (0.1, 0.5, 1.0 and 1.5 mg/L). The two-way ANOVA revealed significant effects of both hormone type ($p < 0.001$) and concentration ($p = 0.04$) on rooting response, although their interaction was not significant ($p = 0.17$) (Table 2). Among the hormones tested, IAA produced the highest rooting efficiency across all concentrations, followed by IBA and NAA. The maximum rooting efficiency (5.00 roots per plantlet) was recorded at 1.5 mg/L IAA, whereas even the lowest concentration (0.1 mg/L) of IAA induced substantial root formation (average = 3.67 roots per plantlet). In contrast, NAA consistently resulted in the lowest rooting efficiency (Figure 3).

A concentration-dependent trend was observed for IAA, with higher concentrations leading to greater root formation (Figure 4). After identifying 1.5 mg/L IAA as the optimal concentration, this treatment was applied to 32 additional genotypes to evaluate rooting performance across genetic backgrounds. Root formation was successfully induced in all genotypes, confirming the broad effectiveness of IAA in promoting root development. Figure 5 shows regeneration and root formation, for example, cultivar Bolivia, illustrating the typical progression through different developmental stages, whereas Figures S1–S6 present similar responses in the remaining 31 genotypes.

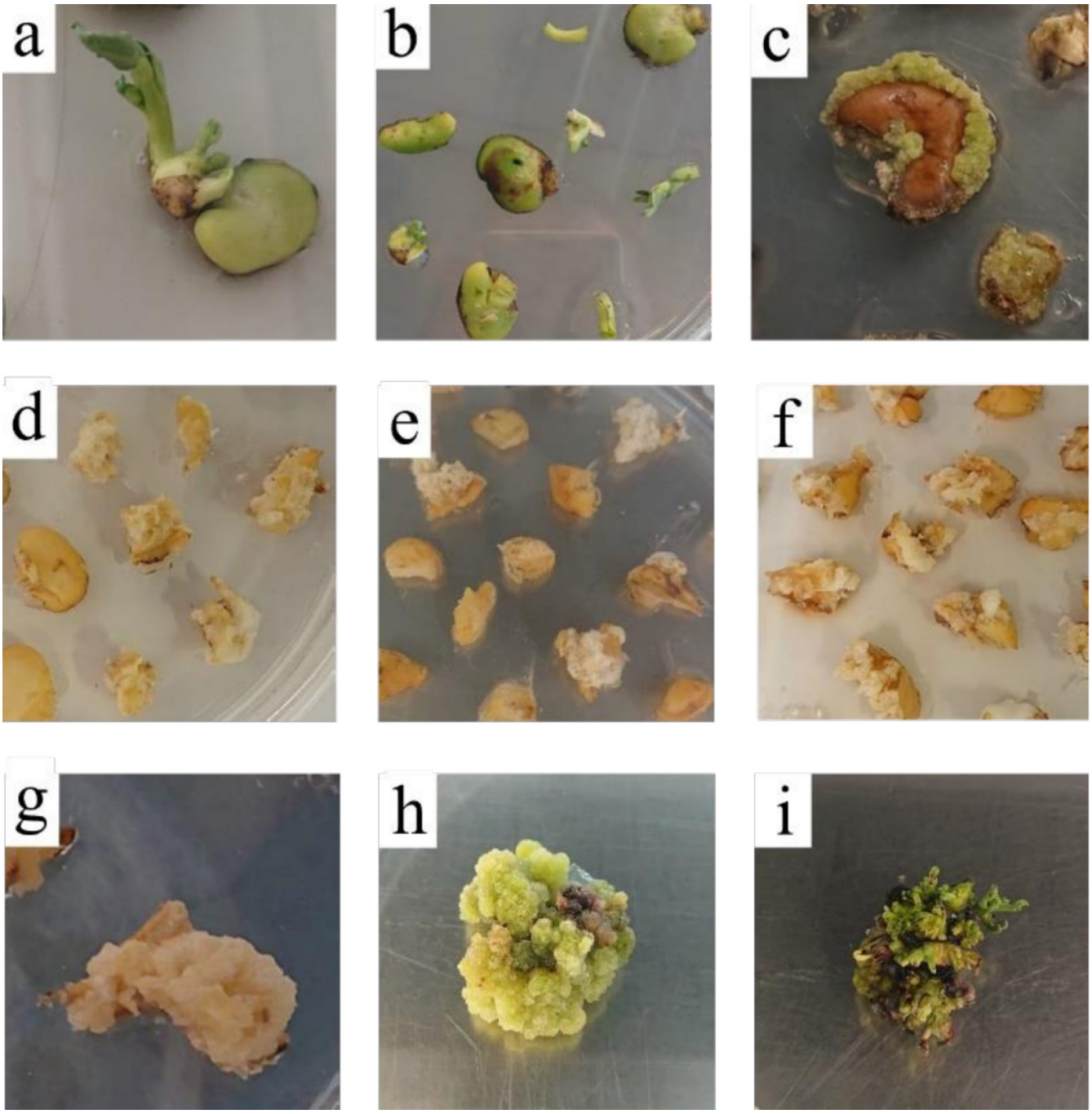


FIGURE 1 | Callus induction and regeneration stages in different genotypes across different medium compositions. (a,b) Direct shoot regeneration under light in cultivar Fuego on MS + 03 and MS + 04 media, showing no callus formation. (c) Callus induction in cultivar Fuego in the dark on MS + 02 medium, producing compact brownish callus. (d) Callus induction in breeding line FB-0110 on MS + 09 medium, yielding friable embryogenic callus. (e,f) Callus induction in breeding line FB-0073 and cultivar Yukon on MS + 02 and MS + 04 media, respectively; MS + 02 produced compact calli, whereas MS + 04 generated soft light-yellow calli. (g) Callus induction in line FB-0073 in the dark on MS + 09 medium. (h) Embryogenic callus formation in FB-0073 on MS + 11 medium. (i) Shoot regeneration from embryogenic callus of genotype FB-0073 on MS + 12 medium. Among tested media, MS + 02 (c,e) effectively initiated healthy callus induction under dark conditions, whereas MS + 09 (d,g) and MS + 11 (h) produced friable embryogenic calli suitable for regeneration. MS + 12 (i) supported optimal shoot regeneration in genotype FB-0073.

3.3 | Establishment of an Efficient Genetic Transformation System for Tiffany and Hedin/2

Transformation was conducted to evaluate the efficiency of the cultivars Tiffany and Hedin/2. GFP fluorescence was detected in embryos 48 h after bombardment, confirming transient gene expression (Figures 6 and 7). PCR amplification of the hygromycin phosphotransferase (*HPT*) gene produced a 735-bp fragment, indicating stable integration of the transgene. Among the tested plants, 151 of 200 (75.5%) Tiffany plants and 8 of 12

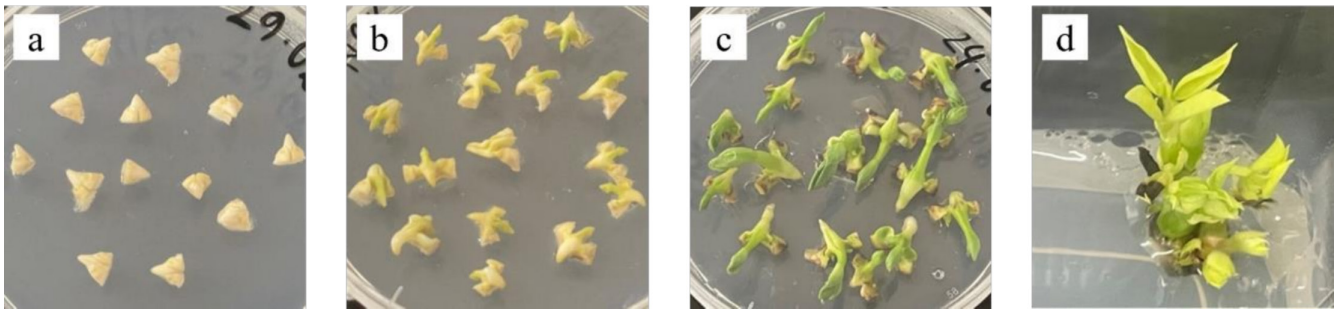


FIGURE 2 | Regeneration stages of cultivar Tiffany on MS1 medium. (a) Freshly extracted embryos from seeds. (b) 2-day-old embryos on MS1 medium. (c) 7-day-old embryos on MS1 medium. (d) Shoot regeneration on MS1 medium, lacking root formation.

TABLE 2 | Two-way ANOVA for the effects of hormone type and concentration on rooting response.

Source	df	*F*	*p*
Hormone	2	12.52	0.00019
Concentration	3	3.24	0.0397
Hormone × concentration	6	1.66	0.174
Residual	24	—	—

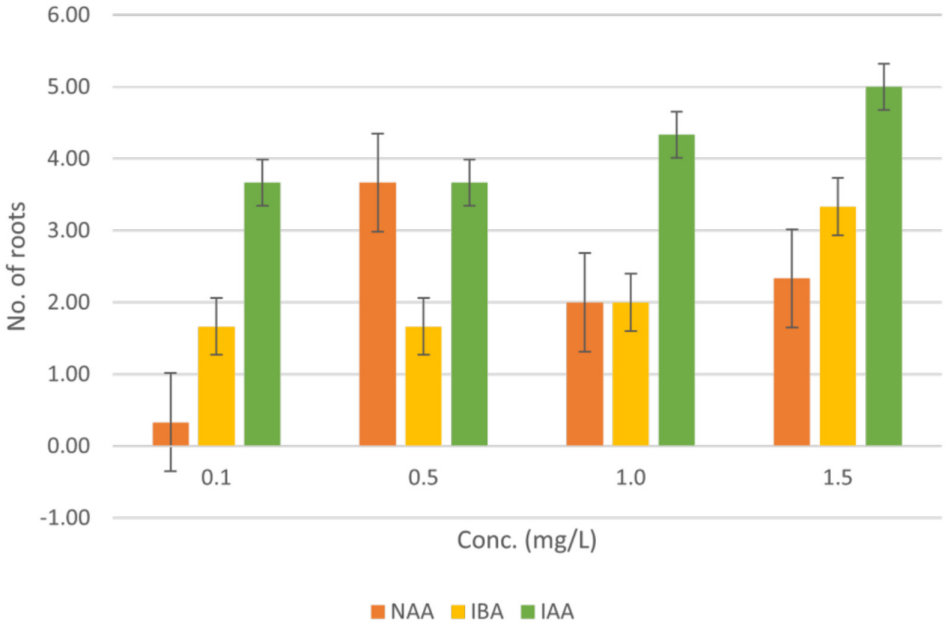


FIGURE 3 | Effect of different hormones on root development in cultivar Tiffany. Root formation in response to varying concentrations (0.1, 0.5, 1.0 and 1.5 mg/L) of NAA, IBA and IAA. Addition of IAA consistently promoted the highest root induction, with optimal response observed at 1.5 mg/L.

(66.7%) Hedin/2 plants were GFP positive. None of the regenerated transgenic plants survived after transfer to soil.

3.4 | Confirmation of Transgene Stability in the T1 Generation

Transformation of 60 embryos from the cultivar Tiffany resulted in the regeneration of 21 plants, of which seven were GFP positive based on PCR analysis, corresponding to a T_0 transformation efficiency of 11.7%. Among these GFP-positive plants, three lines (TG4, TG9 and TG18) produced viable seeds and were advanced to the T1 generation.

In the TG4 line, two seeds were germinated, and both T1 plants exhibited GFP positive and produced a clear 735-bp amplicon in PCR analysis using *hpt*-specific primers, confirming transgene inheritance. Similarly, two T1 plants derived from the TG9 line were GFP positive and yielded the expected 735-bp PCR product. In the TG18 line, one T1 plant was analysed and showed a 735-bp band, confirming stable transgene transmission. Overall, PCR amplification of all five T1 plants verified the presence of

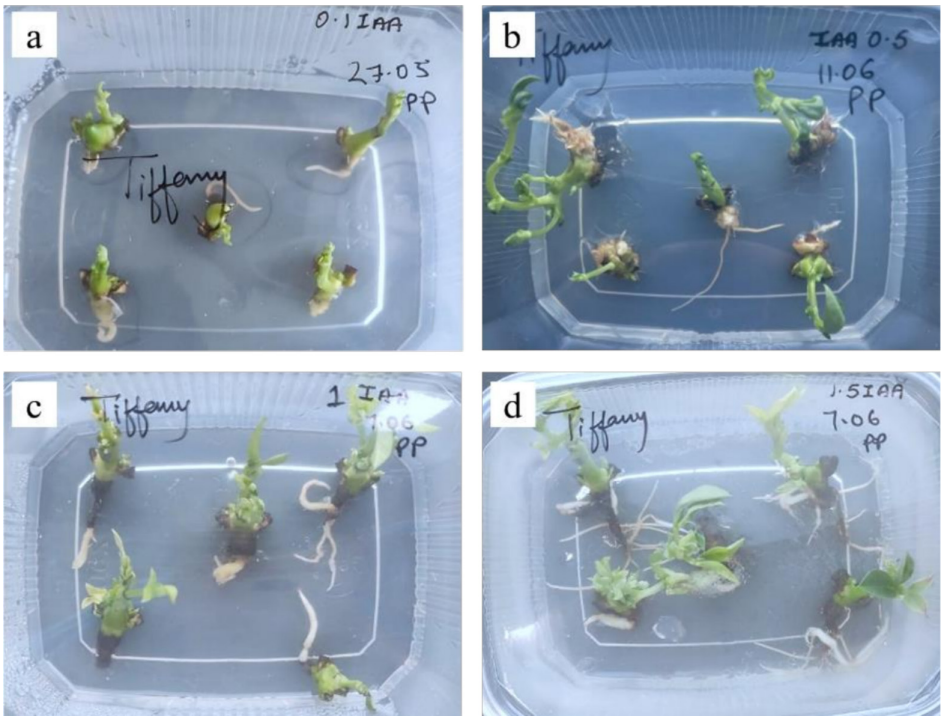


FIGURE 4 | Root formation in cultivar Tiffany on MS2 medium supplemented with different concentrations of indole-3-acetic acid (IAA). Root development with an IAA concentration of (a) 0.1 mg/L, (b) 0.5 mg/L, (c) 1.0 mg/L and (d) 1.5 mg/L.

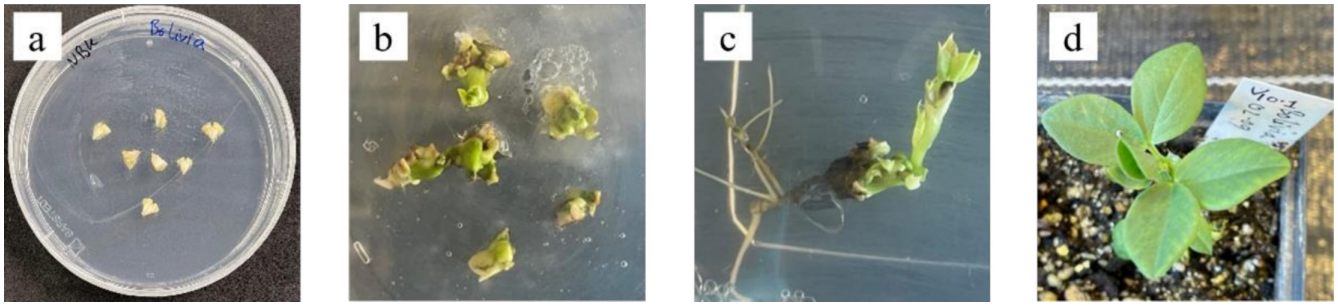


FIGURE 5 | Regeneration and root formation stages in cultivar Bolivia. (a) Excised embryos placed on MS1 medium supplemented with NAA, BAP and KIN (NBK). (b) Germination of embryos on MS1 medium. (c) Root development on MS3 medium supplemented with 1.5 mg/L IAA. (d) Established plants transferred to soil.

the transgene and confirmed stable inheritance from the T0 to the T1 generation (Figure 8).

4 | Discussion

4.1 | Optimised Tissue Culture Media for Regeneration

This study developed an efficient and reliable tissue culture protocol that performed consistently across various genotypes. Direct regeneration was initially accomplished under light conditions in media formulations MS + 02, MS + 04 and MS + 06, however with notable phenolic compounds. Callus induction was enhanced by transferring explants to the same media under dark conditions. Prior research has highlighted the difficulties in optimising media for faba beans, as the synthesis of phenolic compounds may impede tissue culture efficacy (Selva et al. 1989). The establishment of alternative hormone combinations (MS + 09) containing 3 mg/L 2,4-D that stimulated callus induction in previously unresponsive genotypes. This highlights the significance of optimising hormone concentrations to enhance tissue culture responsiveness in recalcitrant species such as faba bean. These findings correspond with existing research on legumes, emphasising the necessity for customised hormone treatments to promote regeneration in recalcitrant cultivars (Bahgat et al. 2009; Ranalli 2003). Additionally, MS1 (Table S4) was found to be effective medium for shoot regeneration in cultivar Tiffany but did not promote root formation, reflecting earlier discoveries in legume tissue culture where the hormonal requirements for shoot and root organogenesis were found to differ considerably (Robert et al. 2023). The challenges in root initiation prompted an investigation into additional hormonal treatments to enhance rooting.

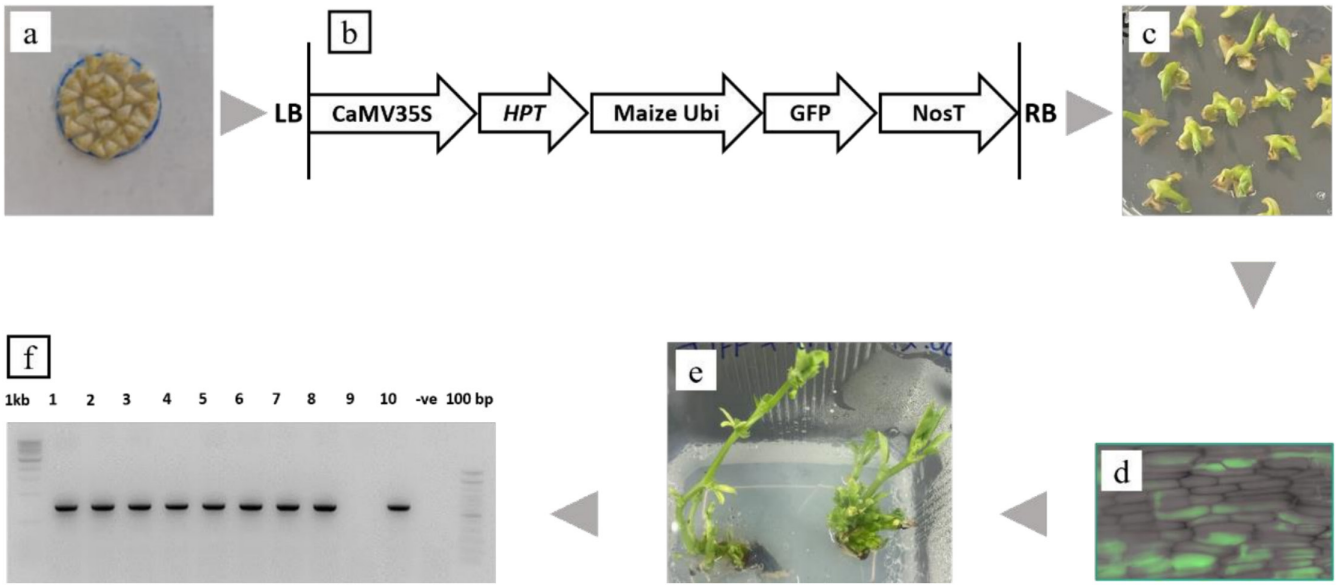


FIGURE 6 | Optimised transformation system cultivar Tiffany using particle bombardment. (a) Embryos placed on MSO medium. (b) Bombardment with the pLH6000 Ubi-GFP construct. (c) Shoot induction on culture medium. (d) GFP expression analysis in transformed tissue. (e) Transgenic plants growing on culture medium. (f) PCR confirmation of transgenic plants using the *hpt* gene.

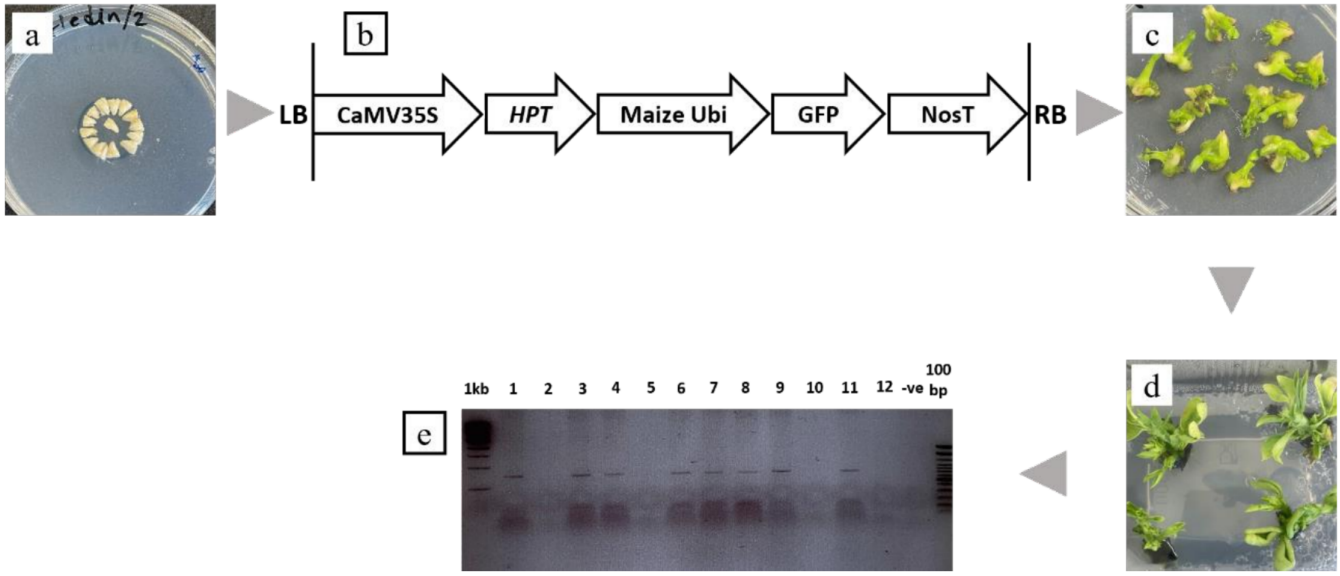


FIGURE 7 | Optimised transformation system for cultivar Hedin/2 using particle bombardment. (a) Embryos placed on MSO medium. (b) Bombardment with the pLH6000 Ubi-GFP construct. (c) Shoot induction on culture medium. (d) Transgenic plants growing on culture medium. (e) PCR confirmation of transgenic plants using the *hpt* gene.

IAA was found to consistently demonstrate superior efficiency for root induction, with optimal results at 1.5 mg/L. Previous reports also showed that IAA is crucial for root initiation in legumes, promoting both root elongation and initiation, even at minimal concentrations (Ismail et al. 2011). IAA is a naturally occurring auxin that modulates cell division and elongation during root development (Anwar et al. 2011; Majda and Robert 2018; Roychoudhry and Kepinski 2022). Besides the hormonal influences on shoot regeneration, alterations in media composition significantly impacted root initiation. For example, reduction in sugar concentration from 30 to 25 g/L significantly improved root formation, reflecting previous reports that reduced sugar in tissue culture media improved rooting in woody plants (Wojtania et al. 2019). Reduced nutrient and sugar concentrations likely alleviate osmotic stress, enhancing hormone absorption and root induction, particularly in conjunction with IAA. Prior studies have highlighted the significance of optimising media composition, especially sucrose concentration, to improve in vitro root development (Gantait and Mukherjee 2021; Ismail et al. 2011). The enhanced root formation in this study suggests that the modified MS2 medium created a more conducive environment for hormonal activity. The efficiency of the optimised IAA concentration, along with the modified media, across 32 additional genotypes demonstrates the robustness and wide applicability of our generalised protocol. The uniformity of root formation

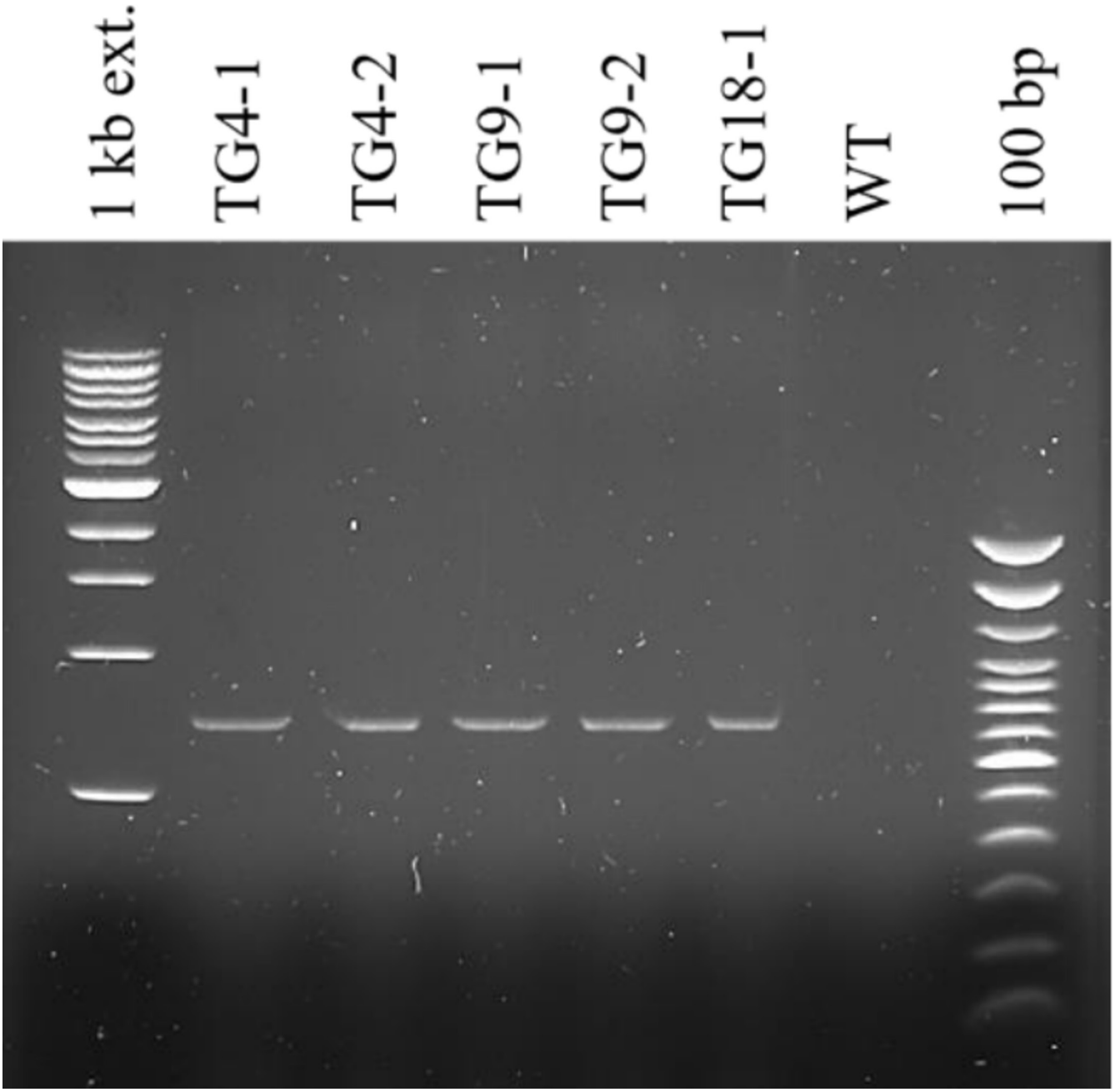


FIGURE 8 | Confirmation of transgene (*hpt* gene) integration in T1 plants using PCR. A 735 bp band corresponding to the *hpt* gene was amplified from genomic DNA extracted from five independent T1 plants. Lanes 1 and 8: DNA ladder; Lanes 2–6: T1 plants showing the expected 735 bp band; Lane 7: Untransformed control.

among various genetic backgrounds tackles a crucial issue in tissue culture, where inconsistent responses to hormonal treatments have frequently constrained regeneration success (Bahgat et al. 2009; Lyu et al. 2021).

4.2 | Advancements in Genetic Transformation for Faba Bean Cultivars

The present study demonstrated an efficient genetic transformation system for cultivars Tiffany and Hedin/2 using particle bombardment. In the initial experiment, 76% of Tiffany embryos and 67% of Hedin/2 plants showed GFP expression following bombardment, indicating successful DNA delivery and expression. However, despite this high transformation rate, the majority of T0 plantlets failed to survive after being transferred to soil. One of the major contributing factors was the incomplete root development observed in many plantlets at the time of transfer, which likely compromised water uptake and nutrient absorption. Additionally, the abrupt transition from in vitro conditions to the greenhouse environment may have caused significant transplant shock and excessive water loss through transpiration, ultimately resulting in high mortality. These findings are consistent with known challenges in legume transformation, where

survival is often hindered by underdeveloped root systems and poor acclimatisation (Desoky et al. 2021; Mangena et al. 2017; Somers et al. 2003).

In contrast, the follow-up experiment, which focused on evaluating transgene inheritance, yielded viable T0 plants that successfully produced seeds. In this experiment, we ensured that plantlets were not transferred to soil until they were fully developed and long roots had formed, significantly improving their survival and establishment. This outcome highlights the critical importance of root development for successful acclimatisation and suggests that extending the in vitro rooting phase prior to soil transfer may substantially enhance survival rates in future transformation efforts.

A further transformation experiment showed a transformation efficiency of 12%, and the T1 progeny confirmed transgene inheritance. These results highlight the stability and heritability of the transgene, supporting the robustness of the developed transformation protocol. Notably, the achieved transformation efficiency remains considerably higher than previous reports in faba bean, which ranged from 2% (Hanafy et al. 2005; Metry 2007) to 6% (Böttinger et al. 2001).

Our transformation protocol was enhanced by optimised tissue culture conditions, specifically the application of MS1 medium enriched with NAA, BAP and KIN for initial growth stimulation, followed by MS2 with IAA for further development. Prior research indicates that the combination of BAP and KIN facilitates shoot organogenesis in legumes, whereas IAA fosters vigorous root development (Akbaş et al. 2011; Ismail et al. 2011; Shekhawat et al. 2015). The modifications to the media were essential for facilitating post-transformation regeneration, which is often a key constraint in transformation (Anwar et al. 2011; Böttinger et al. 2001; Gantait and Mukherjee 2021). The successful transformation we achieved in two different cultivars illustrates the scalability and reliability of our protocol, with potential applications in other cultivars and possible transfer to other legume species. Collectively, these findings validate the reliability of the optimised transformation protocol, highlighting its potential for genetic improvement efforts in faba bean breeding programmes.

5 | Conclusions

This study established a comprehensive and optimised protocol for the regeneration and genetic transformation of faba beans. An optimised media composition effectively enhanced shoot regeneration and root formation across 33 evaluated genotypes, indicating an extensive applicability of the protocol. Healthy tissue culture–grown plants could be readily transferred and acclimatised in a greenhouse, highlighting the practical utility of the protocol in breeding programmes. Transformation through particle bombardment led to the development of GFP-positive plants, and heritable transgene integration was confirmed in T1 plants, validating the stability of transformation achieved through this protocol. This approach demonstrates strong potential for the introduction of novel genetic alterations into superior faba bean varieties. Ultimately, the protocol can potentially contribute to accelerating breeding progress, which have been recalcitrant to tissue culture regeneration.

Author Contributions

Md Mamunur Rashid: investigation, visualization, formal analysis, writing – original draft. **Paridhi Paridhi:** investigation, resources. **Sai Aravind Mandava:** investigation, resources. **Marina Grölz:** investigation, resources. **Babette Knoblauch:** investigation, resources. **Stavros Tzigos:** investigation, resources. **Rod J. Snowdon:** conceptualization, funding acquisition, supervision, writing – review and editing. **Sruthy Maria Augustine:** conceptualization, methodology, project administration, supervision, validation, writing – review and editing (supporting).

Acknowledgements

The work was supported by DFG grant 469336000 to R.J.S. from the German Research Society (DFG) for the International Research Training Group IRTG 2843 Accelerating Crop Genetic Gain. The authors thank Norddeutsche Pflanzenzucht Hans-Georg Lembke KG (NPZ, Hohenlieth, Germany) for providing seeds of the elite breeding lines used in the study. Open Access funding enabled and organized by Projekt DEAL.

Funding

This research was supported by the Deutsche Forschungsgemeinschaft (DFG, German Research Society) under grant number 469336000, as part of the International Research Training Group (IRTG) 2843, "Accelerating Crop Genetic Gain."

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Data will be made available on request.

References

Akbaş, F., Ç. Işıkalan, S. Namli, P. Orcan, and D. Basaran. 2011. "Direct Plant Regeneration From In Vitro-Derived Leaf Explants of *Hypericum spectabile*, a Medicinal Plant." *Journal of Medicinal Plant Research* 5: 217–2181.

Anwar, F., S. S. Alghamdi, M. H. Ammar, and K. H. M. Siddique. 2011. "An Efficient In Vitro Regeneration Protocol for Faba Bean (*Vicia faba* L.)." *Journal of Medicinal Plant Research* 5, no. 28: 6460–6467. https://doi.org/10.5897/JMPR11.146.

Bahgat, S., O. A. Shabban, O. El-Shihy, D. A. Lightfoot, and H. A. El-Shemy. 2009. "Establishment of the Regeneration System for *Vicia faba* L." *Current Issues in Molecular Biology* 11, no. s1: 47–54. https://doi.org/10.21775/cimb.011.i47.

Bangar, S. P., and P. Kajla. 2022. "Introduction: Global Status and Production of Faba-Bean." In *Faba Bean: Chemistry, Properties and Functionality*, edited by S. Punia Bangar and S. Bala Dhull, 1–15. Springer International Publishing. https://doi.org/10.1007/978-3-031-14587-2_1.

Bean, S. J., P. S. Gooding, P. M. Mullincaux, and D. R. Davies. 1997. "A Simple System for Pea Transformation." *Plant Cell Reports* 16, no. 8: 513–519. https://doi.org/10.1007/BF01142315.

Böttinger, P., A. Steinmetz, O. Schieder, and T. Pickardt. 2001. "*Agrobacterium*-Mediated Transformation of *Vicia faba*." *Molecular Breeding* 8, no. 3: 243–254. https://doi.org/10.1023/A:1013711210433.

Chaudhary, V., P. Kajla, and Shobhit. 2022. "Chemistry, Nutrient Composition and Quality of Faba Beans." In *Faba Bean: Chemistry,*

Properties and Functionality, edited by S. Punia Bangar and S. Bala Dhull, 75–96. Springer International Publishing. https://doi.org/10.1007/978-3-031-14587-2_4.

De Cillis, F., B. Leoni, M. Massaro, M. Renna, and P. Santamaria. 2019. "Yield and Quality of Faba Bean (*Vicia faba* L. Var. Major) Genotypes as a Vegetable for Fresh Consumption: A Comparison Between Italian Landraces and Commercial Varieties." *Agriculture* 9, no. 12: 253. https://doi.org/10.3390/agriculture9120253.

Desoky, E.-S. M., E. Mansour, E.-S. E. A. El-Sobky, et al. 2021. "Physio-Biochemical and Agronomic Responses of Faba Beans to Exogenously Applied Nano-Silicon Under Drought Stress Conditions." *Frontiers in Plant Science* 12: 637783. https://doi.org/10.3389/fpls.2021.637783.

Doyle, J. J., and J. L. Doyle. 1990. "Isolation of Plant DNA From Fresh Tissue." *Focus* 12: 13–15.

Federal Plant Variety Office. 2024. "Descriptive Variety List: Cereals, Maize, Oil and Fibre Crops, Legumes, Beets, Cover Crops 2024." Hannover, Germany: Federal Plant Variety Office. https://www.bundessortenamt.de.

Gantait, S., and E. Mukherjee. 2021. "Tissue Culture-Based Genetic Improvement of Fava Bean (*Vicia faba* L.): Analysis on Previous Achievements and Future Perspectives." *Applied Microbiology and Biotechnology* 105, no. 18: 6531–6546. https://doi.org/10.1007/s00253-021-11517-2.

Hanafy, M., T. Pickardt, H. Kiesecker, and H.-J. Jacobsen. 2005. "*Agrobacterium*-Mediated Transformation of Faba Bean (*Vicia faba* L.) Using Embryo Axes." *Euphytica* 142, no. 3: 227–236. https://doi.org/10.1007/s10681-005-1690-4.

Imani, J., and K.-H. Kogel. 2020. "Plant Transformation Techniques: *Agrobacterium*- and Microparticle-Mediated Gene Transfer in Cereal Plants." In *Biolistic DNA Delivery in Plants: Methods and Protocols*, edited by S. Rustgi and H. Luo, 281–294. Springer US. https://doi.org/10.1007/978-1-0716-0356-7_15.

Ismail, R. M., H. E. M. Elazab, G. M. H. Hussein, and E. A. Metry. 2011. "In Vitro Root Induction of Faba Bean (*Vicia faba* L.)." *GM Crops* 2, no. 3: 176–181. https://doi.org/10.4161/gmcr.2.3.18003.

Jayakodi, M., A. A. Golicz, J. Kreplak, et al. 2023. "The Giant Diploid Faba Genome Unlocks Variation in a Global Protein Crop." *Nature* 615, no. 7953: 652–659. https://doi.org/10.1038/s41586-023-05791-5.

López-Bellido, F. J., L. López-Bellido, and R. J. López-Bellido. 2005. "Competition, Growth and Yield of Faba Bean (*Vicia faba* L.)." *European Journal of Agronomy* 23, no. 4: 359–378. https://doi.org/10.1016/j.eja.2005.02.002.

Lyu, J. I., R. Ramekar, J. M. Kim, et al. 2021. "Unraveling the Complexity of Faba Bean (*Vicia faba* L.) Transcriptome to Reveal Cold-Stress-Responsive Genes Using Long-Read Isoform Sequencing Technology." *Scientific Reports* 11: 21094. https://doi.org/10.1038/s41598-021-00506-0.

Majda, M., and S. Robert. 2018. "The Role of Auxin in Cell Wall Expansion." *International Journal of Molecular Sciences* 19, no. 4: 951. https://doi.org/10.3390/ijms19040951.

Mangena, P., P. W. Mokwala, and R. V. Nikolova. 2017. "Challenges of In Vitro and In Vivo *Agrobacterium*-Mediated Genetic Transformation in Soybean." In *Soybean – The Basis of Yield, Biomass and Productivity*, edited by M. Kasai. InTech. https://doi.org/10.5772/66708.

Metry, E. A. 2007. "Regeneration and Microprojectile-Mediated Transformation in *Vicia faba* L." *Arab Journal of Biotechnology* 10: 23–36.

Mookkan, M. 2018. "Particle Bombardment – Mediated Gene Transfer and GFP Transient Expression in *Seteria viridis*." *Plant Signaling & Behavior* 13, no. 4: e1441657. https://doi.org/10.1080/15592324.2018.1441657.

Murashige, T., and F. Skoog. 1962. "A Revised Medium for Rapid Growth and Bio Assays With Tobacco Tissue Cultures." *Physiologia Plantarum* 15, no. 3: 473–497. https://doi.org/10.1111/j.1399-3054.1962.tb08052.x.

R Core Team. 2025. "R: A Language and Environment for Statistical Computing." R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org.

Ranalli, P. 2003. "Breeding Methodologies for the Improvement of Grain Legumes." In *Improvement Strategies of Leguminosae Biotechnology*, edited by P. K. Jaiwal and R. P. Singh, 3–21. Springer Netherlands. https://doi.org/10.1007/978-94-017-0109-9_1.

Robert, H. S., M. G. Heisler, L. Xiao, and B. Landrein. 2023. "Editorial: The Hormonal Control of Shoot Organogenesis and Morphogenesis: From Leaves to Seeds." *Frontiers in Plant Science* 14: 1319859. https://doi.org/10.3389/fpls.2023.1319859.

Roychoudhry, S., and S. Kepinski. 2022. "Auxin in Root Development." *Cold Spring Harbor Perspectives in Biology* 14, no. 4: a039933. https://doi.org/10.1101/cshperspect.a039933.

Sanford, J. C., F. D. Smith, and J. A. Russell. 1993. "Optimizing the Biolistic Process for Different Biological Applications." In *Methods in Enzymology*, vol. 217, 483–509. Academic Press. https://doi.org/10.1016/0076-6879(93)17086-K.

Schiemann, J., and G. Eisenreich. 1989. "Transformation of Field Bean (*Vicia faba* L.) Cells: Expression of a Chimaeric Gene in Cultured Hairy Roots and Root-Derived Callus." *Biochemie und Physiologie der Pflanzen* 185, no. 1: 135–140. https://doi.org/10.1016/S0015-3796(89)80170-2.

Selva, E., B. Stouffs, and M. Briquet. 1989. "In Vitro Propagation of *Vicia faba* L. by Micro-Cutting and Multiple Shoot Induction." *Plant Cell, Tissue and Organ Culture* 18, no. 2: 167–179. https://doi.org/10.1007/BF00047742.

Shekhawat, M. S., N. Kannan, M. Manokari, and C. P. Ravindran. 2015. "In Vitro Regeneration of Shoots and Ex Vitro Rooting of an Important Medicinal Plant *Passiflora foetida* L. Through Nodal Segment Cultures." *Journal of Genetic Engineering and Biotechnology* 13, no. 2: 209–214. https://doi.org/10.1016/j.jgeb.2015.08.002.

Somers, D. A., D. A. Samac, and P. M. Olhoft. 2003. "Recent Advances in Legume Transformation." *Plant Physiology* 131: 892–899. https://doi.org/10.1104/pp.102.017681.

Wojtania, A., E. Skrzypek, and A. Marasek-Ciolakowska. 2019. "Soluble Sugar, Starch and Phenolic Status During Rooting of Easy- and Difficult-to-Root Magnolia Cultivars." *Plant Cell, Tissue and Organ Culture (PCTOC)* 136, no. 3: 499–510. https://doi.org/10.1007/s11240-018-01532-z.

Youseif, S. H., F. H. Abd El-Megeed, and S. A. Saleh. 2017. "Improvement of Faba Bean Yield Using *Rhizobium*/*Agrobacterium* Inoculant in Low-Fertility Sandy Soil." *Agronomy* 7, no. 1: 2. https://doi.org/10.3390/agronomy7010002.

Zhuo, Q., and X. Yang. 2004. "Methods of Hygromycin B Phosphotransferase Activity Assay in Transgenic Plant." *Journal of Hygiene Research* 33, no. 4: 502–504.

Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Table S1:** Composition of culture media used for callus induction and regeneration. **Table S2:** Callus induction response of different genotypes under light and dark conditions. **Table S3:** PCR amplification protocol. **Table S4:** Composition of MS1 medium. **Table S5:** Composition of MS3 medium. **Table S6:** Composition of MS medium. **Table S7:** Composition of MS2 medium. **Figure S1:** Growth stages of various genotypes, including (a) Trumpet, (b) Victus, (c) Macho, (d) FB-0073, (e) FB-0110 and (f) FB-0130, showing sequential stages of development from initial culture to advanced growth with root formation. **Figure S2:** Growth stages of various genotypes, including (a) Yukon, (b) FB-0206, (c) FB-0052, (d) Dosis, (e) Allison, and (f) Hedin/2, showing sequential stages of development from initial culture to advanced growth with root formation **Figure S3:** Growth stages of various genotypes, including (a) FB-0131, (b) Lynx,

(c) Expresso, (d) Alfaed, (e) Loki and (f) FB-0125, showing sequential stages of development from initial culture to advanced growth with root formation. **Figure S4:** Growth stages of various genotypes, including (a) FB-0129, (b) FB-0136, (c) DL-Nevado, (d) Hiverna-Sublinie, (e) FB-0134 and (f) Kristal, showing sequential stages of development from initial culture to advanced growth with root formation. **Figure S5:** Growth stages of various genotypes, including (a) FB-0132, (b) FB-0137, (c) Pantani, (d) Mallory, (e) Scirocco and (f) FB-0050, showing sequential stages of development from initial culture to advanced growth with root formation. **Figure S6:** Growth stages of genotype (a) FB-0121, showing sequential stages of development from initial culture to advanced growth with root formation.