

In Vitro Callus Induction and Morphogenesis in Soybean (*Glycine max* L.) Using Optimized Plant Growth Regulators

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ABSTRACT

Soybean (*Glycine max* L.) is an economically important leguminous crop valued for its high protein and oil content. The present study aimed to establish an efficient protocol for in vitro callus induction and morphogenesis using Murashige and Skoog (MS) medium supplemented with different concentrations of plant growth regulators. Cotyledonary node, hypocotyl and leaf explants were surface sterilized and cultured under aseptic conditions. Maximum callus induction was observed on MS medium containing 2.0 mg L⁻¹ 2,4-dichlorophenoxyacetic acid (2,4-D), producing friable cream-colored callus within 10–15 days. Compact green callus with morphogenic potential developed after transfer to regeneration medium containing 2.0 mg L⁻¹ BAP and 0.5 mg L⁻¹ NAA. Among the explants tested, cotyledonary nodes exhibited the highest callus induction frequency followed by hypocotyls and leaves. Morphogenesis was strongly influenced by the type of explant and hormonal balance. The study demonstrates that optimized auxin-cytokinin combinations significantly improve soybean tissue culture efficiency and provide a reliable platform for rapid propagation, genetic transformation and crop improvement.

Keywords: Soybean, *Glycine max*, Plant tissue culture, Callus induction, Morphogenesis, 2,4-D, BAP, MS medium.

1. INTRODUCTION

Soybean (*Glycine max* L.) is one of the world's most important oilseed crops, contributing significantly to global food security due to its high protein (38–42%) and oil (18–22%) contents. Besides its nutritional importance, soybean improves soil fertility through symbiotic nitrogen fixation and serves as an important model crop for plant biotechnology.

Plant tissue culture provides a powerful approach for rapid multiplication, germplasm conservation, genetic transformation and development of disease-resistant cultivars. Among tissue culture techniques, callus induction and subsequent morphogenesis are critical steps for successful plant regeneration. Callus consists of undifferentiated proliferating cells capable of regenerating complete plants under suitable hormonal conditions.

Murashige and Skoog (1962) medium remains the most widely used nutrient medium for tissue culture because of its balanced mineral composition. Auxins, particularly 2,4-D, stimulate cell division and callus formation, whereas cytokinins such as BAP promote shoot differentiation and organogenesis. The ratio of auxin to cytokinin determines the developmental pathway of cultured tissues (Skoog and Miller, 1957).

Soybean has traditionally been considered a recalcitrant species for tissue culture because regeneration efficiency varies considerably among genotypes and explants. Nevertheless, improvements in culture media and plant growth regulator

combinations have significantly enhanced regeneration efficiency during the last decade. Efficient regeneration protocols are indispensable for genome editing, *Agrobacterium*-mediated transformation and molecular breeding.

Therefore, the present investigation aimed to optimize callus induction and morphogenesis from soybean explants cultured on MS medium supplemented with different concentrations of plant growth regulators.

2. MATERIALS AND METHODS

Plant Material

Healthy seeds of soybean (*Glycine max* L.) were collected from Vilasrao Deshmukh College of Agricultural Biotechnology, Latur.

Surface Sterilization

Seeds were washed under running tap water for 20 min, treated with 70% ethanol for 30 seconds followed by 0.1% mercuric chloride (or 2% sodium hypochlorite) for 5 minutes and rinsed three to four times with sterile distilled water.

2.3 Culture Medium

The basal culture medium used for callus induction and morphogenesis was the Murashige and Skoog (MS) medium, which is widely recognized for supporting the *in vitro* growth and regeneration of plant tissues. The medium was supplemented with 3% (w/v) sucrose as a carbon source and solidified with 0.8% (w/v) agar. The pH of the medium was adjusted to 5.8 before sterilization. The prepared medium was dispensed into culture vessels and sterilized by autoclaving at **121°C for 20 min under 15 psi pressure**. After cooling to approximately 45–50°C, filter-sterilized plant growth regulators were added according to the experimental treatments before the medium was poured into sterile culture vessels.

2.4 Plant Growth Regulators

To optimize callus induction and subsequent shoot regeneration, different concentrations and combinations of auxins and cytokinins were incorporated into the MS medium. For callus induction, **2,4-dichlorophenoxyacetic acid (2,4-D)** was tested at concentrations ranging from **0.5 to 3.0 mg L⁻¹**. For shoot regeneration and morphogenesis, **6-benzylaminopurine (BAP)** at **1.0–3.0 mg L⁻¹** was combined with **α -naphthaleneacetic acid (NAA)** at **0.2–1.0 mg L⁻¹**. The hormone combinations were selected based on their reported effectiveness in promoting cellular dedifferentiation, organogenesis, and regeneration in soybean tissue culture.

2.5 Culture Conditions

Surface-sterilized explants, including cotyledonary nodes, hypocotyls, and leaf segments, were aseptically inoculated onto the prepared culture media under laminar airflow conditions. The cultures were incubated in a growth chamber maintained at **25 ± 2°C** with a **16 h light/8 h** dark photoperiod. Illumination was provided by cool white fluorescent lamps at a light intensity of approximately **2500 lux**. Cultures were observed regularly for contamination, callus initiation, proliferation, and morphogenic development, and subculturing was carried out at appropriate intervals to maintain vigorous growth.

2.6 Data Recording

The cultured explants were monitored throughout the experimental period, and observations were recorded at regular intervals to evaluate the efficiency of different hormonal treatments. The parameters assessed included the number of days required for callus initiation, percentage of callus induction, callus colour, callus texture, fresh weight of callus, morphogenic response, **and** shoot regeneration frequency. These observations were used to identify the most suitable

explant type and plant growth regulator combination for efficient callus induction and plant regeneration in *Glycine max* L.

Statistical Analysis

Experiments were conducted in triplicate. Data were expressed as Mean $\pm$ SD and analyzed using one-way ANOVA followed by Tukey's test at $P < 0.05$.

3. RESULTS

The response of soybean (*Glycine max* L.) explants to in vitro culture was significantly influenced by both the type of explant and the concentration of plant growth regulators incorporated into the Murashige and Skoog (MS) medium. Callus initiation was first observed within **10–15 days** of inoculation, beginning at the wounded regions of the explants. The induced callus initially appeared creamy-white and compact, gradually becoming friable with continued proliferation under suitable hormonal conditions.

Among the explants evaluated, cotyledonary node explants exhibited the highest callus induction frequency ($92.0 \pm 1.5\%$), followed by hypocotyl explants ($81.0 \pm 2.4\%$) and leaf explants ($68.0 \pm 2.8\%$). Cotyledonary node-derived calli were vigorous, friable, and proliferated rapidly, whereas leaf-derived calli showed comparatively slower growth and a compact texture.

The concentration of 2,4-dichlorophenoxyacetic acid (2,4-D) markedly affected callus induction (Table 1). No callus formation was observed on the control medium lacking growth regulators. The addition of **0.5 mg L^{-1} 2,4-D** induced compact callus in $52.0 \pm 2.1\%$ of explants, while increasing the concentration to **1.0 mg L^{-1}** significantly enhanced callus induction ($71.0 \pm 1.8\%$). The highest callus induction (**$92.0 \pm 1.5\%$**) was achieved on MS medium supplemented with **2.0 mg L^{-1} 2,4-D**, producing abundant cream-coloured, friable callus with high proliferative capacity. Further increasing the concentration to **3.0 mg L^{-1}** slightly reduced callus induction ($84.0 \pm 2.4\%$) and resulted in partial browning of the tissue, indicating the inhibitory effect of excessive auxin.

Following four weeks of culture, actively growing calli were transferred to regeneration media containing different concentrations of benzylaminopurine (BAP) and α -naphthaleneacetic acid (NAA). Distinct morphogenic changes were observed within two weeks of transfer. The callus gradually changed from cream to green, became compact, and developed numerous nodular structures, which subsequently differentiated into shoot primordia.

Among the regeneration treatments, MS medium supplemented with 2.0 mg L^{-1} BAP and 0.5 mg L^{-1} NAA produced the highest shoot regeneration frequency (**$76.0 \pm 2.2\%$**), followed by 3.0 mg L^{-1} BAP + 0.5 mg L^{-1} NAA ($69.0 \pm 1.9\%$). Medium containing 2.0 mg L^{-1} BAP alone resulted in comparatively lower regeneration ($54.0 \pm 2.6\%$), indicating that the synergistic interaction between cytokinin and auxin was essential for efficient organogenesis.

Overall, the results demonstrate that MS medium supplemented with 2.0 mg L^{-1} 2,4-D is optimal for callus induction, whereas 2.0 mg L^{-1} BAP combined with 0.5 mg L^{-1} NAA provides the most effective hormonal combination for shoot regeneration and morphogenesis in soybean.

Table 1. Effect of different concentrations of 2,4-D on callus induction in soybean (*Glycine max* L.)

Treatment	Callus induction (%)	Callus characteristics
Control	0.0 ± 0.0	No response
MS + 0.5 mg L^{-1} 2,4-D	52.0 ± 2.1	Compact, cream
MS + 1.0 mg L^{-1} 2,4-D	71.0 ± 1.8	Cream, moderately friable
MS + 2.0 mg L^{-1} 2,4-D	92.0 ± 1.5	Cream, friable, rapidly proliferating
MS + 3.0 mg L^{-1} 2,4-D	84.0 ± 2.4	Brownish, friable

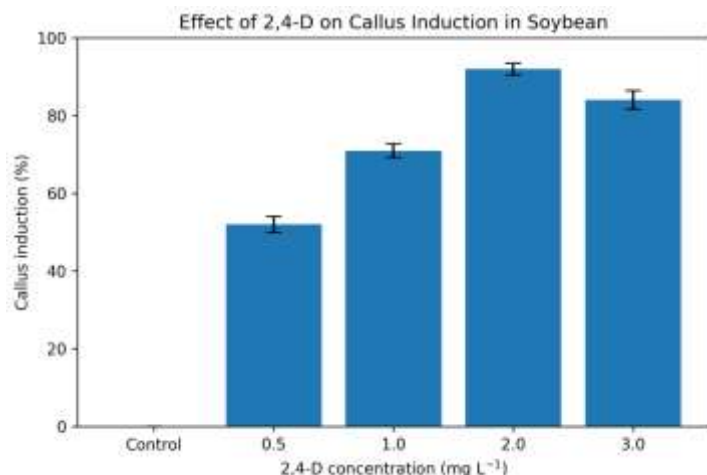


Figure 1. Effect of different concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) on callus induction (%) in *Glycine max* cultured on Murashige and Skoog (MS) medium. Values represent **mean $\pm$ SD (n = 3)**. Maximum callus induction was observed at **2.0 mg L⁻¹ 2,4-D**, while no callus formation occurred in the control treatment.

Table 2. Effect of growth regulators on morphogenesis and shoot regeneration

Regeneration medium	Shoot regeneration (%)	Morphogenic response
MS + 2.0 mg L ⁻¹ BAP + 0.5 mg L ⁻¹ NAA	76.0 $\pm$ 2.2	Green compact callus with multiple shoot buds
MS + 3.0 mg L ⁻¹ BAP + 0.5 mg L ⁻¹ NAA	69.0 $\pm$ 1.9	Moderate shoot formation
MS + 2.0 mg L ⁻¹ BAP	54.0 $\pm$ 2.6	Limited shoot regeneration

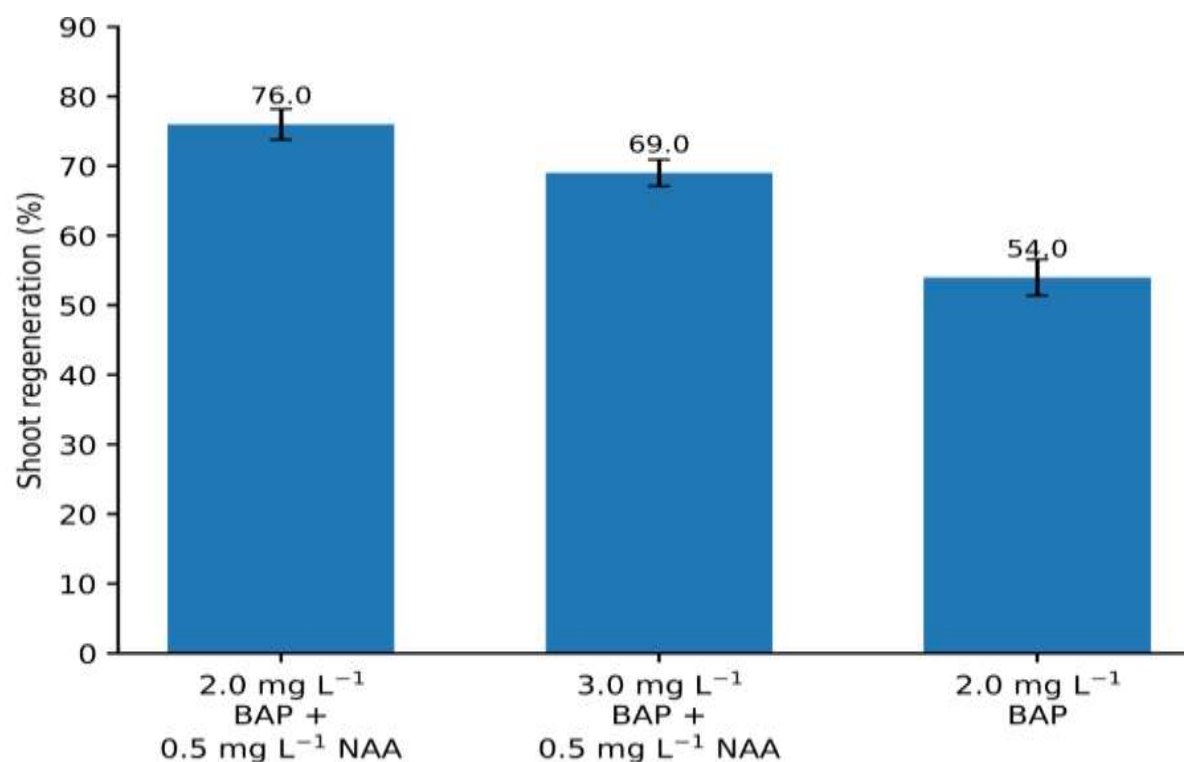


Figure 2. Effect of different regeneration media on shoot regeneration in *Glycine max*. Bars represent the mean shoot regeneration frequency (%), and error bars indicate the standard deviation ($\pm$ SD; n = 3). The highest regeneration was

obtained on MS medium supplemented with 2.0 mg L⁻¹ BAP + 0.5 mg L⁻¹ NAA, followed by 3.0 mg L⁻¹ BAP + 0.5 mg L⁻¹ NAA and 2.0 mg L⁻¹ BAP.

4. DISCUSSION

The present investigation established an efficient in vitro regeneration protocol for *Glycine max* using cotyledonary node, hypocotyl, and leaf explants cultured on MS medium supplemented with different concentrations of plant growth regulators. Among the auxin treatments evaluated, **2.0 mg L⁻¹ 2,4-D** proved to be the most effective for callus induction, producing rapidly proliferating, friable callus with high morphogenic competence. Similar observations have been reported in soybean and other legumes, where 2,4-D promotes cellular dedifferentiation by activating auxin-responsive transcription factors involved in cell cycle re-entry and acquisition of pluripotency (Ikeuchi et al., 2013; Xu et al., 2022).

The superior regenerative response of cotyledonary node explants compared with hypocotyl and leaf tissues may be attributed to their greater meristematic activity and higher endogenous hormonal balance. Juvenile tissues generally possess enhanced regenerative competence due to the presence of actively dividing cells, making them more responsive to exogenous growth regulators. Similar findings have been reported by Joyner et al. (2010), who identified cotyledonary nodes as the most suitable explant for soybean regeneration.

Callus characteristics varied considerably with auxin concentration. Cream-coloured, friable calli obtained on 2.0 mg L⁻¹ 2,4-D exhibited excellent regenerative potential, whereas higher auxin concentrations induced tissue browning and reduced morphogenesis. Browning is commonly associated with phenolic oxidation and loss of cellular viability, which adversely affects regeneration efficiency in soybean tissue culture.

Successful shoot regeneration was achieved after transferring proliferating calli to regeneration medium containing 2.0 mg L⁻¹ BAP and 0.5 mg L⁻¹ NAA. The synergistic interaction between cytokinin and auxin promoted the transition from undifferentiated callus to organized shoot meristems. Cytokinins stimulate shoot apical meristem formation, while low concentrations of auxin maintain cellular competence during organogenesis. The present findings are consistent with recent reports demonstrating enhanced shoot regeneration on BAP-NAA combinations in soybean tissue culture.

Recent molecular studies have further revealed that auxin-mediated activation of ARF7, ARF19, LBD16, LBD17, and LBD18 genes regulates cellular reprogramming and callus formation, whereas cytokinin signalling controls shoot meristem development through the activation of WUSCHEL and SHOOT MERISTEMLESS pathways (Frontiers in Plant Science, 2023; Alok et al., 2025). The regeneration efficiency obtained in the present study therefore supports current molecular evidence explaining hormone-regulated morphogenesis in soybean. The optimized regeneration system developed in this investigation provides a reliable platform for rapid clonal propagation, genetic transformation, genome editing, germplasm conservation, and soybean improvement programmes, thereby contributing to future applications in plant biotechnology and sustainable crop enhancement.

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