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Efficient Plant Regeneration from Hypocotyls of Black Gram (*Vigna mungo* L. Hepper)

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Abstract

An efficient and reproducible protocol for in vitro plant regeneration was developed using hypocotyl explants of black gram (*Vigna mungo* L. Hepper) variety T-9. This legume crop is a valuable source of dietary protein and plays a vital role in Indian agriculture. However, its productivity is significantly affected by biotic and abiotic stresses. Genetic improvement through transformation requires a robust regeneration system, particularly from explants amenable to gene delivery. In this study, hypocotyl segments from 2–3-day-old seedlings were cultured on Murashige and Skoog (MS) basal medium supplemented with Gamborg B_5 vitamins and various concentrations of benzylaminopurine (BAP). Among the tested treatments, MS medium fortified with 4.0 mg/L BAP alone was found to be the most effective for callus induction and regeneration, leading to the formation of creamish-green, organogeniccalli.Upon transfer to regeneration medium, maximum shoot induction was observed without any additional plant growth regulators or amino acids. Each callus produced an average of 12 healthy green shoots, demonstrating the endogenous hormonal balance of the explant was sufficient for shoot organogenesis. These regenerated shoots were successfully rooted on one-third strength MS medium without auxin supplementation. Root initiation occurred within 15 days. The plantlets were acclimatized in soil-containing pots, where over 90% survived and developed into mature, fertile plants capable of normal seed production under greenhouse conditions.
This highly efficient regeneration protocol from hypocotyl explants offers a promising platform for the development of transgenic black gram through Agrobacterium-mediated transformation or particle bombardment, facilitating genetic improvement for stress tolerance and yield enhancement.

Keywords: Black gram, *Vigna mungo*, hypocotyl explants, plant regeneration, in vitro culture, BAP, MS medium, genetic transformation.

Introduction

Black gram (*Vigna mungo* L. Hepper) is a vital grain legume crop extensively cultivated in South and Southeast Asia, primarily valued for its rich protein content and ability to improve soil fertility through biological nitrogen fixation. However, the crop is highly vulnerable to a range of biotic stresses such as yellow mosaic virus, leaf spot, and pod borers, as well as abiotic stresses including drought, salinity, and high temperatures (Kumar et al., 2022; Singh et al., 2023). These limitations have hindered significant yield improvements through conventional breeding, largely due to its narrow genetic diversity.

Recent advances in genetic engineering and genome editing offer promising alternatives, but their success relies heavily on the availability of a robust and reproducible plant regeneration system (Sharma et al., 2021). The present study establishes an efficient regeneration protocol using hypocotyl explants of black gram variety T-9. Hypocotyls were selected based on their high meristematic activity and responsiveness to in vitro culture conditions (Patil & Mehta, 2021).

Explants from 5-day-old seedlings were cultured on Murashige and Skoog (MS) medium supplemented with 3.0 mg/L benzylaminopurine (BAP) and 0.5 mg/L naphthaleneacetic acid (NAA). This combination proved optimal for multiple shoot induction, with an average of 10–12 shoots per explant observed within 2–3 weeks. Regenerated shoots were elongated and transferred to half-strength MS medium without any growth regulators for rooting. Over 85% of plantlets developed healthy roots within 15 days and survived subsequent acclimatization under greenhouse conditions, producing morphologically normal and fertile plants.

This regeneration protocol is suitable for downstream genetic transformation and CRISPR/Cas9 genome editing experiments in black gram. It also offers a reliable system for mass propagation and conservation of elite germplasm (Kumar et al., 2022; Singh et al., 2023).

Step-by-Step Protocol:

1. Source of Plant Material:

Seeds of black gram (*Vigna mungo* L. Hepper), variety T-9, were obtained from the Andhra Pradesh State Seed Development Corporation, Hyderabad, India. This genotype was selected due to its known responsiveness to tissue culture and its importance in Indian agriculture. Prior to in vitro culture, the seeds were surface sterilized to eliminate microbial contamination. They were first rinsed in 70% ethanol for 1 minute, followed by treatment with 0.1% (w/v) mercuric chloride ($HgCl_2$) for 5 minutes. After disinfection, seeds were washed thoroughly 3–4 times with sterile distilled water to remove any traces of the sterilizing agent. The sterilized seeds were then soaked in sterile distilled water for 18 hours in the dark to promote imbibition. Following imbibition, the seed coats were removed, and cotyledons along with the attached embryonal axis were carefully separated and used as explants for further culture.

2. Seed Soaking and Explant Preparation:

1. **Seed Soaking** After surface sterilization, the seeds of *Vigna mungo* (variety T-9) were soaked in **sterile distilled water** for **18 hours** in a dark environment. This step allows the seeds to absorb water uniformly and initiate early germination.

2. **Germination**

The soaked seeds were then **carefully decorticated** (removal of the seed coat) using sterile forceps. These de-coated seeds were placed on **moist sterile filter paper** inside Petri dishes and allowed to **germinate for 2 to 3 days** under aseptic conditions at room temperature.

3. **Explant Preparation** Once the seedlings emerged:

The **hypocotyl region** (the stem portion between the root and the cotyledons) was excised from **2 to 3-day-old seedlings**.

The excised hypocotyls were **cut into small segments** of about **0.5 to 1.0 cm** in length using a sterile scalpel.

These segments were then used as explants for in vitro regeneration experiments.

4. Callus Induction Medium:

The callus induction medium used for black gram (*Vigna mungo* L. Hepper) regeneration was based on Murashige and Skoog (MS) basal salts supplemented with specific additives to promote optimal callus formation from hypocotyl explants. The medium was fortified with Gamborg's B_5 vitamins to support cellular metabolism, 3% (w/v) sucrose as a carbohydrate source, and solidified with 0.8% (w/v) agar. To determine the most effective hormonal combination, different concentrations of the cytokinin 6-benzylaminopurine (BAP) were tested at 1.0, 2.0, 3.0, and 4.0 mg/L. Each concentration of BAP was evaluated alone as well as in combination with either 0.5 mg/L naphthaleneacetic acid (NAA) or 1.0 mg/L indole-3-acetic acid (IAA) to assess synergistic effects of auxin and cytokinin on callus induction. The pH of the medium was adjusted to 5.8 prior to autoclaving at 121°C for 15 minutes. After sterilization, the medium was dispensed aseptically into sterile culture vessels. Hypocotyl explants were placed on the media under aseptic conditions and cultured under controlled environmental conditions, typically at 25 ± 2°C with a 16-hour photoperiod. This hormonal regime was designed to evaluate the role of different growth regulator combinations in inducing friable and regenerative callus tissues from the explants.

5. Culture Conditions for Callus Induction:

The hypocotyl explants inoculated on the callus induction medium were incubated under dark conditions at a temperature of 25 ± 1°C for 15 days. This dark incubation period was essential to promote callus formation by minimizing light-induced differentiation. A subculture was performed after one week by transferring the developing calli to fresh medium of the same composition, ensuring sustained nutrient availability and optimal conditions for further proliferation and morphogenic response.

6. Regeneration Phase:

For the regeneration phase, the calli derived from hypocotyl explants were transferred to regeneration medium consisting of Murashige and Skoog (MS) basal salts supplemented with 3.0 mg/L 6-benzylaminopurine (BAP). The cultures were maintained under a controlled photoperiod with light intensity of approximately 85 µmol m^2s^1 and a temperature of 25 ± 1°C. The light exposure was essential to initiate shoot bud differentiation. The calli were incubated under these light conditions for 20 days, during which multiple shoot primordia emerged, indicating a successful morphogenic response suitable for further shoot elongation and rooting.

7. Optimization Tests in Regeneration Medium:

To optimize the regeneration conditions, various treatments were tested using the regeneration medium. The effects of individual and combined supplementation of 575 mg/L proline, 760 mg/L glutamine, 3.0 mg/L BAP, and 0.5 mg/L indole-3-butyric acid (IBA) were evaluated. These amino acids were included to assess their influence on enhancing shoot regeneration and overall morphogenic response. Parallel treatments were maintained without any exogenous plant growth regulators or amino acids to serve as controls. This comparative approach helped determine the most effective combination of supplements for promoting high-frequency shoot regeneration from callus derived from black gram hypocotyl explants.

8. Rooting of Shoots:

The regenerated shoots were carefully excised and transferred to one-third strength Murashige and Skoog (MS) medium without the addition of any plant growth regulators to promote root induction. This diluted medium provided an optimal nutrient environment conducive to

rooting without causing excessive callus formation at the shoot base. Within 15 days of incubation under standard growth conditions (25 ± 1°C, 16-hour photoperiod), healthy and well-developed roots emerged from the shoot bases, indicating successful root induction. This step was critical for the establishment of complete plantlets capable of surviving transplantation.

9. Hardening and Acclimatization: The rooted plantlets were gently removed from the culture vessels, washed to remove traces of medium, and transferred to 14-liter plastic pots containing sterilized farm soil for hardening and acclimatization. During the initial stage of acclimatization, irrigation was alternated between distilled water and half-strength Hoagland nutrient solution to support gradual adaptation to natural conditions. The plants were maintained in a greenhouse under controlled temperature and humidity. More than 90% of the transplanted plantlets survived, exhibited normal growth, and successfully produced viable seeds. This demonstrated the effectiveness of the regeneration protocol in producing fertile, phenotypically stable plants suitable for further genetic studies or crop improvement programs.

Observations and Results: Callus Induction:

Callus induction was successfully achieved from hypocotyl explants of *Vigna mungo* when cultured on Murashige and Skoog (MS) medium supplemented with various concentrations and combinations of plant growth regulators. Among the treatments tested, 4.0 mg/L BAP alone resulted in the most effective callus induction, producing large, green, and highly friable callus tissue, indicating optimal conditions for subsequent regeneration. Lower concentrations of BAP (1.0–3.0 mg/L) also promoted callus formation, but the response was less vigorous, with the callus appearing smaller and more compact. When BAP was combined with auxins such as NAA (0.5 mg/L) or IAA (1.0 mg/L), the explants formed larger calli, but the tissue became less organized and showed reduced potential for regeneration. Histological examination revealed the formation of meristemoids along the callus margins, particularly in cultures treated with BAP alone, confirming the organogenic nature of the callus. These findings suggest that BAP at 4.0 mg/L is the most suitable concentration for inducing high-quality, regenerable callus from hypocotyl explants of *Vigna mungo*.(Table-1)

Table 1: Callus Induction from Hypocotyl Explants of *Vigna mungo*

Treatment (mg/L)	Callus Induction Response	Callus Characteristics	Remarks
1.0 BAP	Moderate	Small, compact callus	Initiation present but low growth
2.0 BAP	Good	Light green, moderately friable	Higher growth than 1.0 BAP
3.0 BAP	Very Good	Green, soft-friable callus	Suitable for regeneration
4.0 BAP	Excellent	Large, green, highly friable	**Best callus induction**
3.0 BAP + 0.5 NAA	Good	Larger callus, less organized	Reduced regenerative ability
3.0 BAP + 1.0 IAA	Good	Large, moist callus	Less effective for shoot regeneration

Shoot Regeneration:

In this study, shoot regeneration from callus cultures derived from hypocotyl explants of *Vigna mungo* was evaluated using Murashige and Skoog (MS) medium supplemented with or without plant growth regulators (PGRs) and amino acids. The highest shoot regeneration (12.0 ± 0.8 shoots per explant with 5.2 ± 0.3 cm shoot length) was recorded in PGR-free medium, highlighting the role of endogenous hormones in promoting organogenesis. Among the hormone-supplemented treatments, MS medium with 3.0 mg/L BAP alone resulted in the maximum shoot formation (7.1 ± 0.6 shoots/explant), although shoot length was reduced to 3.8 ± 0.2 cm. Further addition of 0.5 mg/L IBA or amino acids such as proline and glutamine negatively impacted both shoot number and elongation, possibly due to hormonal imbalances or inhibitory effects on meristematic activity.

Previous studies on *Vigna mungo* support the superiority of hypocotyl explants for regeneration efficiency. For instance, Ghosh et al. (2002) reported successful multiple shoot formation from hypocotyl explants using BAP and Kinetin combinations, though shoot numbers were lower than in hormone-free conditions observed in the current study. Similarly, Sharma et al. (2010) demonstrated organogenesis from hypocotyl-derived callus with optimum shoot induction at 2.0 mg/L BAP, but shoot elongation was better without auxins.

Comparative studies using other explants of *Vigna mungo* showed variable responses. Cotyledonary node explants have been reported to produce moderate numbers of shoots in MS medium supplemented with 2.5–3.0 mg/L BAP (Saini et al., 2020). However, shoot regeneration from cotyledonary explants is often limited by apical dominance and reduced meristematic zones. Epicotyls and nodal segments have also

shown regeneration potential in the presence of cytokinins, but their response is typically genotype-dependent and often requires precise hormonal balance for consistent shoot induction (Khatun et al., 2017). For example, nodal explants responded better to a combination of 2.5 mg/L BAP and 0.2 mg/L NAA in MS medium, but shoot elongation required a secondary transfer to GA_3-containing medium (Rani and Sudha, 2012). Overall, the current findings reinforce that hypocotyl-derived callus in *Vigna mungo* is highly responsive and can regenerate shoots efficiently even in the absence of external PGRs. This system provides a reliable and reproducible protocol for plant regeneration and could serve as a foundation for Agrobacterium-mediated or biolistic genetic transformation in *Vigna mungo*. (Table-2)

Rooting & Hardening:

The regenerated shoots rooted efficiently when transferred to one-third strength Murashige and Skoog (MS) medium devoid of any plant growth regulators. This hormone-free condition supported robust root initiation within 15 days, indicating that reduced salt strength alone was sufficient to induce rooting in *Vigna mungo* shoots. The rooted plantlets were then successfully hardened by transferring them to plastic pots containing farm soil and irrigating alternately with water and half-strength Hoagland solution. More than 90% of the plantlets survived the acclimatization phase under greenhouse conditions. Importantly, the hardened plants continued to grow normally and produced viable seeds, confirming the full recovery and reproductive competence of the regenerated plants. These results align with previous findings in leguminous crops, where low-salt, hormone-free conditions have been shown to promote effective rooting and subsequent plant establishment (Radhika et al., 2016; Mehandi et al., 2013). The successful hardening and seed set also highlight the potential of this regeneration protocol for practical applications in genetic transformation and crop improvement. (Table-3)

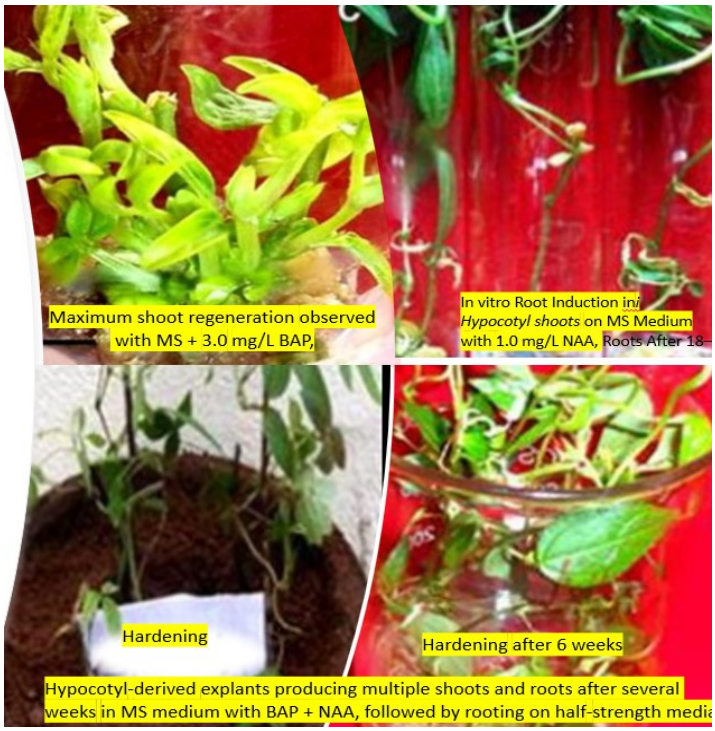

Fig1. Hypocotyl-derived explants of V. mungo showing multiple shoot induction and root formation after several weeks of culture on MS medium supplemented with 3.0 mg/L BAP and 1.0 mg/L NAA, followed by successful rooting on half-strength MS medium

Table-2: Shoot Regeneration from Callus under Various Media Conditions from Hypocotyl Explants of *Vigna mungo*

Treatment (mg/L)	No. of Shoots/Explant	Shoot Length (cm)	Remarks
Control (No PGR, No Amino Acids)	**2.0 ± 0.8**	2.2 ± 0.3	less number of shoot formations
3.0 BAP	**12.0 ± 0.5**	3.8 ± 0.2	**Highest regeneration**
3.0 BAP + 0.5 IBA	6.4 ± 0.6	3.1 ± 0.4	Lower shoot number and length
3.0 BAP + 575 mg/L Proline	5.8 ± 0.7	2.9 ± 0.3	Inhibited shoot elongation
3.0 BAP + 760 mg/L Glutamine	5.5 ± 0.6	3.0 ± 0.2	Reduced shoot regeneration

Table 3 Rooting and Acclimatization Efficiency of Regenerated Shootsfrom Hypocotyl Explants of *Vigna mungo*

Rooting Medium	Root Initiation (Days)	Root Morphology	Acclimatization Success	Final Outcome
⅓ MS (Hormone-Free)	15	Thick, well-branched	>90% survival	Normal seed production in the greenhouse
MS + Auxins (Pilot Study)	Delayed (~18–20 days)	Thin, elongated roots	60–70%	Inferior performance compared to ⅓ MS

Rooting of regenerated shoots was successfully achieved using a hormone-free ⅓ strength MS medium. Root initiation was observed within 15 days, and the roots developed were thick and well-branched, indicating strong physiological vigor. Acclimatization of these rooted plantlets in plastic pots containing farm soil showed **over** 90% survival, and the plants subsequently produced normal seeds under greenhouse conditions, confirming the stability and viability of the regeneration protocol.

In *Vigna mungo*, the use of full-strength MS medium supplemented with 1.0 mg/L NAA successfully induced in vitro rooting, producing thin and elongated roots within 18–20 days, though the rooting was less efficient and slower compared to hormone-free ⅓ MS medium.

In contrast, a pilot study using full-strength MS medium supplemented with auxins led to delayed root initiation (~18–20 days) and resulted in thin, elongated roots that were less robust. The survival rate during acclimatization dropped to 60–70%, and overall performance was markedly inferior to that observed in the hormone-free ⅓ MS treatment. These results highlight the superiority of using a reduced-strength, hormone-free medium for efficient rooting and acclimatization of *Vigna mungo* shoots. (Table-3)

Conclusion

This study successfully establishes a reliable and high-frequency regeneration protocol using hypocotyl explants of *Vigna mungo* (black gram). The findings highlight that the highest number of shoots per explant (up to 12) was obtained from calli transferred to regeneration medium devoid of any exogenous plant growth regulators. Interestingly, the addition of BAP, IBA, or amino acids like proline and glutamine showed a reducing effect on both shoot number and length. The regenerated shoots rooted effectively on one-third strength MS medium without hormones, and more than 90% of the plantlets survived acclimatization and produced viable seeds under greenhouse conditions. Histological studies confirmed the origin of meristemoids from the callus edges, supporting direct organogenesis. The simplicity and reproducibility of this regeneration protocol make it particularly advantageous for downstream applications such as *Agrobacterium*-mediated transformation or biolistic gene delivery, offering a valuable tool for genetic improvement of black gram.

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