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PROTOCOL OPTIMIZATION FOR EMBRYO RESCUE: IMMATURE EMBRYO CULTURE OF WHEAT AND VARIETAL PERFORMANCE ANALYSIS FOR CALLUS INDUCTION

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Abstract *Wheat (Triticum aestivum L.) is one of the most important staple crops worldwide, which is now under threat of productivity loss caused by changing climates, genetic erosion, and increasing biotic and abiotic stresses. The development of genetic and biotechnological solutions depends greatly on the establishment of highly efficient cell culture and plant regeneration systems. In this study, the protocol for tissue culture propagation of embryo rescue was optimized, and the callus induction efficiency of 4 different wheat genotypes, Sehar-2006, Galaxy-13, Sawera-2024, and Falak-2025, was assessed. Immature embryos were dissected and surface sterilized with a sequential treatment of sodium hypochlorite, Tween-20, and 70% ethanol. Murashige and Skoog (MS) basal medium containing 30 g L^{-1} sucrose, 2 g L^{-1} Phytagel, and 3 mL L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) stock solution was used for the culture of the sterilized explants. After a 2-week incubation period under controlled environmental conditions, there were considerable genotypic differences in terms of callus induction performance. Falak-2025 showed the highest callus induction and embryo rescue success rate (~78%), which was followed by Sawera-2024 with callus induction of ~70% and embryo rescue of ~50%, whereas Galaxy-13 showed callus induction of ~45% and embryo rescue of ~45%. The results obtained validate that the immature embryos are a good explant source because of their high metabolic activity and high regeneration potential. In addition, the application of the exogenous auxin 2,4-D was essential for the initiation of embryogenic callus formation. Finally, the optimization of all the above-mentioned aspects (genotypic selection, stage of explant culture, and hormonal balance) plays a crucial role in the success of wheat embryo culture. The improved protocol described in this study offers a reliable and repeatable callus induction system that could be used in subsequent wheat improvement projects, such as genetic transformation and producing stress-tolerant cultivars.*

Keywords: *Immature Embryo; Callus Media; 2,4-D; MS Media*

Introduction

Wheat (*Triticum aestivum L.*) is a member of the Poaceae family, and it is the most widely grown agronomic crop and is one of the basic food crops in the world. Climate volatility, new crop diseases, agricultural pests, and rapidly growing global populations of humans approaching 8 billion are all having a dramatic impact on current wheat production and demand. (Mohammed and Baldwin, 2024) At the same time, the genetic diversity of the current wheat varieties is decreasing over time, as a result of continuous monocultures and genetic erosion in the conventional breeding process. It is imperative to imbibe resilient and robust genes into the crop genome to achieve yield protection against the severe biotic and abiotic stresses in the evolving environmental conditions. (Alshaharni et al., 2024; Mohammed and Baldwin, 2024)

Reliable in vitro plant regeneration from cultured cells and tissues is a key to the future of biotechnological interventions for crop improvement. Therefore, good callus culture systems and efficient plant regeneration procedures are essential in tissue culture research. The choice of the explant source is crucial in determining the percentage of callus induction and plantlet regeneration in wheat tissue culture. In previous tissue culture studies, explants from immature leaves, immature inflorescences, mature embryos, seeds, apical meristems, and immature embryos have been used. Of these, wheat is the embryo culture system that consistently produces the greatest percentages of callus and plant regeneration. Therefore, there is a consensus that the optimal explant source to maximize regeneration efficiency is immature embryos. (Özgen et al., 1998; Tamimi and Othman, 2021).

Although maturely superior, use of immature embryos has some logistical disadvantages, as they are difficult to acquire all year round, as they reach the stage for culture at the optimum environmental window of opportunity. But the culture of immature embryos is still a vital method for producing haploid wheat plants that can be doubled to produce 100% homozygosity in a single generation. This technique has also been used to overcome dormancy, determine seed viability, propagate rare wild species, and enable the insertion of target genes by genetic transformation. (Ahmadpour et al., 2016; Mohammed and Baldwin, 2024; Özgen et al., 1998)

The regeneration potential of wheat cultivars is influenced by a variety of factors that include genotype screening, genetic backgrounds, inoculation methods, explant pre-treatments, culture incubation conditions, basal media compositions, and specific phytohormone concentrations. However, there were significant interactions between explant type and genotype, as well as between the different ingredients in the medium that determined the morphology and embryogenic capacity of the callus derived from them. Previous reports show that callus growth from immature embryos can be stimulated by the addition of 3.5 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) to MS medium, whereas specific mixtures of kinetin, BAP, and tyrosine have been reported to be effective for the regeneration of mature embryos. (Chopra et al., 2022; Mayerni et al., 2020) Maximizing metabolic activity by the use of certain growth configurations is required since mature differentiated tissues have a reduced ability to re-enter active cell division compared to metabolically active immature tissues. Thus, systematic studies of hormone balance and genotypic response are needed to further develop wheat tissue culture methods. (Ahmadpour et al., 2016; Alshaharni et al., 2024; Mohammed and Baldwin, 2024)

This study aimed to assess callus induction response of four different cultivars of wheat crop using an optimized immature embryo rescue system as a baseline system to create somaclonal variations and for the genetic transformation process.

Materials and methods

Plant Material and Explant Collection

The seeds of 4 major varieties of wheat named Sehar-2006, Galaxy-13, Sawera-2024,a and Falak-2025 were chosen for the explant extraction. The maternal plants were cultivated in an open field, and spikes were harvested when the plants were in the transitioning physiological stage, which was characterized by a greenish-yellow color. Plant materials were all collected from the specialized nursery and crossing blocks of the Agricultural Biotechnology Research Institute (ABRI), Ayub Agricultural Research Institute (AARI), Faisalabad. Spikes were collected, threshed manually, and seeds isolated in Petri dishes, genotype labelled.

Surface Sterilization Protocols

Sterile seed cultures were established by surface sterilizing the isolated seeds according to the following strict procedure in a horizontal laminar airflow chamber (Table 1 Figure 1):

- A 1–2 drop surfactant (Tween-20) in a solution of 5% (5ml/100ml) commercial sodium hypochlorite (Bleach) and 95% (95ml/100ml) sterile distilled water was made fresh.
- The seeds were soaked in this solution for 20–25 minutes in a sterile flask with agitation in continuous motion, inside which the flask was wrapped with aluminum foil.
- After the removal of the bleaching solution, the seeds were chemically sterilized by being immersed for exactly 1 minute in 70% (70ml/100ml) ethanol in the laminar flow chamber, and then the ethanol was decanted.
- The sterilized seeds were washed three times with autoclaved distilled water for 30 seconds each to remove all chemical residues.
- Afterwards, the seeds were placed on sterile filter paper to remove excess moisture on the seed surface before the embryos were cut out.

Table 1: Sterilization Stock Solution

Sterilization Solution (100 ml)		
Sr. No.	**Ingredients**	**Quantity**
1	Distilled Water	95 ml
2	Sodium Hypochlorite (Bleach)	5 ml
3	Tween 20%	1-2 Drops

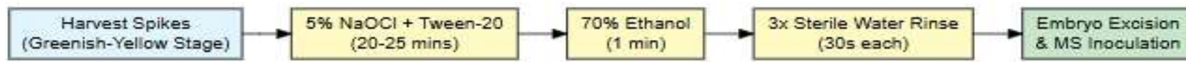

Figure 1. Flow Diagram: Explant Collection to Sterilization to Inoculation

Preparation of Callus Induction Medium

The culture medium was made up from standard Murashige and Skoog (MS) basal formulations with the following composition (Table 2):

- The granular ingredients, such as D-sucrose (30 g/L) and MS basal salts (4.43 g/L), were weighed using a calibrated digital analytical balance.
- Then the ingredients were dissolved in a beaker of distilled water with 3 mL L^{-1} of 2,4-D hormone solution.
- The pH of this core solution was checked and/or adjusted to a known pH range of 5.7-5.8 by the addition of dilute hydrochloric acid (HCl) to lower the alkalinity or sodium hydroxide (NaOH) to raise the alkalinity.
- **Table 2: Ingredients for Culture Medium**

Callus Media (Growth Media)		
Sr. No.	**Ingredients**	**Quantity**
1	Distilled Water	1 L
2	D-Sucrose	30 g
3	MS Media	4.43 g
4	2,4-D	3 ml
5	Phytagel	2g
6	pH (HCl and NaOH)	5.7-5.8

Aseptic Excision and Culture Inoculation

Flame-sterilized surgical scalpel blades and forceps were used for isolating the immature embryos within the laminar flow hood. A single seed was sterilized, and the morphological tip, where the immature embryo is found, which is macroscopically visible as a clear whitish area, was cut off at a diagonal angle with forceps. The immature embryo was exposed, carefully removed, and placed directly into the prepared culture vessels. Each embryo transfer was separated by the heat sterilization of the scalpel to prevent cross-contamination.

Two containment methods were used: One embryo each in separate test tubes; 15 embryos in separate Petri dishes with uniform distribution. Culture tubes were tightly wrapped with polypropylene sheets and

- The other 500 mL of distilled water was put into a container, and 2 g L^{-1} of Phytagel (the solidifying gelling agent) was suspended and dissolved in this.
- The two components were then combined and well mixed to make a total compounded volume of 1000 mL (1L).
- The medium flask was covered with aluminium foil and sterilized under standard autoclaving conditions (121℃ at 15 psi).
- After sterilization, the molten medium was allowed to cool a little and poured under aseptic conditions into sterile Petri dishes and test tubes in the laminar flow chamber.
- Petri plates and tubes were left to set, covered with plastic paraffin wrap, and kept in a clean incubation place until they were inoculated.

securely bound with rubber bands, while Petri dishes were completely wrapped with wrapping tape. All culture vessels were properly labelled according to the specific cultivar genotype name, name of the operator and the date of inoculation. Finally, clean paper was placed over the cultured vessels to mimic dark/low light conditions and the vessels were moved to a climate-controlled incubation room for continuous culture for 2 weeks to allow for the callus to be initiated.

Results and discussion

After the two weeks of incubation on the optimized MS medium configuration, the immature embryo explants were analyzed to evaluate the callus induction response. Significant variation in tissue culture responsiveness was recorded between the evaluated germplasm.

The genotypic performance in terms of embryo rescue and callus induction success rate was distributed as (Figure 2):

- Falak-2025 showed the best tissue culture efficiency with a success rate of embryo rescue around 78%.
- Sawera-2024: Showed the second highest capacity of about 70% of success.
- Sehar-2006: Gave a moderate response, success rate ~50%.
- Galaxy-13: Had the lowest response of the cultivars tested with a success rate of around 45%.

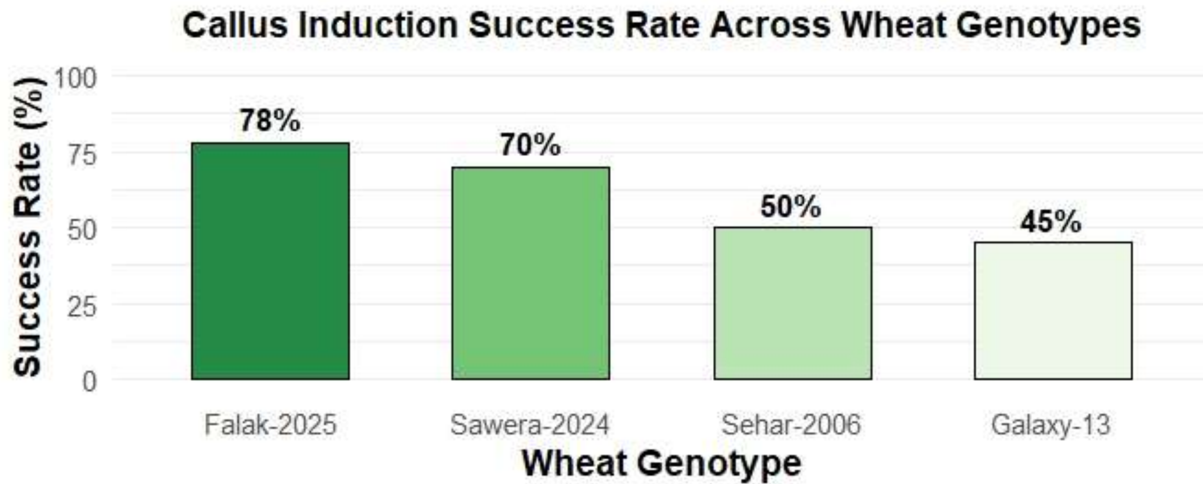

Figure 2. Callus induction percentage

The results obtained here show that a combination of several factors, such as the selection of the genotype, the developmental stage of the explants and hormone composition, is important for successful in vitro culture of wheat embryos. Results here show that immature embryos behave better than older embryos, which is consistent with the results that have been reported in the past for which juvenile tissues are regarded as highly reactive explants. Their high tissue culture efficiency is due to their high metabolic status and high dedifferentiation and proliferation capacity of their cells. An immature embryo is the best standard explant for high-frequency embryogenic callus development, while a mature embryo can be used as an alternative under specific configurations (Figure 3).

Figure 3. Callus induction at different stages

Dedifferentiation was triggered by an exogenous application of the synthetic auxin 2,4-D at 3 mL L-1 (from stock solution) in this work. This new and unorganized mass of cells is called a callus and is caused by primary biochemical signals (auxins, especially 2,4-D), which interfere with normal somatic tissue programming and stimulate rapid cell division. The large differences in performance obtained between the four varieties suggest that the endogenous hormone balance and genetic background of modern Pakistani wheat lines have a major influence on the response of the cells to exogenous growth regulators. The high percentage of responses received from recently released cultivars, like Falak-2025 and Sawera-2024, emphasizes their role as potential lines for further advanced biotechnological improvement trials.

Conclusion

In this study, an optimized protocol of tissue culture for high-efficiency embryo rescue and callus induction in wheat (*Triticum aestivum L.*) is established. The in vitro embryo rescue procedure is successful when immature seeds are taken from the greenish-yellow transitional stage, and a sequential

surface sterilization procedure is utilized with sodium hypochlorite, Tween-20, and 70% ethanol under aseptic conditions. The modern cultivar Falak-2025 was the most responsive cultivar based on embryo rescue and callus initiation success rate (~78%). Differentiation was greatly promoted by the empirical medium matrix comprising MS basal salts at 4.43 g L^{-1}, with the addition of 30 g L^{-1} sucrose and 3 mL L^{-1} of 2,4-D stock solution, and this could be achieved within 2-weeks. The optimized protocol is a reliable platform for the production of embryogenic calli for effective application in downstream genetic transformation, somaclonal variation exploitation, and breeding for stress resistance in wheat.

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Declaration

Data Availability statement

All authenticated data have been included in the manuscript.

Consent for publication

Not applicable

Declaration of Competing Interests

The authors declare that they have no conflict of interest.

Author Contribution Statement

Muhammad Abdul Rehman Khalid conducted research under supervision of Muhammad Waqas Jamil and Hussnain Chaudhary and wrote article. Muhammad Abdul Rehman Khalid and Muawaz Ul Hassan make final editing was carried out and approved for final publication.

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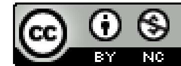
Informed Consent

Not applicable.

Ethical Statement

Not applicable



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