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Effect of cold treatment and growth regulators in plant regeneration via in-vitro anther culture in rice (*Oryza sativa* L.)



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ABSTRACT

In-vitro androgenesis offers a distinctive approach for generating homozygous doubled-haploid plants. Utilizing haploid plant breeding speeds up the process of obtaining doubled haploid plants, which plays a crucial role in rice breeding. This research aims to enhance the production of doubled haploids through In-vitro anther culture in rice and to select doubled haploid plants with desirable traits. The research finds that a 9-day cold treatment positively influences callus formation when using modified N6 media, whereas MS media is applied for plant regeneration. Six plant regeneration media (PRM1, PRM2, PRM3, PRM4, PRM5, and PRM6) were tested, with PRM5 (BAP 0.5 mg/L, NAA 0.25 mg/L) and PRM6 (BAP 1.5 mg/L, NAA 1.0 mg/L, kinetin 1.0 mg/L) with successful plant regeneration. PRM6 showed the highest regeneration rate with 12 plants, while PRM5 resulted in 8 plants. Other media (PRM1–PRM4) led only to callus blackening, with no plant regeneration. A total of 20 albino plants were developed, demonstrating that higher growth regulator concentrations may increase callus regrowth under these conditions. A major challenge observed in this work was the complete regeneration of albino plants, indicating impaired chloroplast development. The study contributes to rice androgenesis research by identifying effective cold pre-treatment durations, evaluating the hormonal combinations for callus proliferation and regeneration, and highlighting critical bottlenecks such as albinism that must be addressed in future protocol optimization.

Keywords: Anther culture, MS media, N6(modified), Albino, Double Haploid, Phytohormone, Callus, Rice, Tissue culture, Androgenesis, Speed breeding, Plant regeneration, Homozygous, In-vitro.

1. Introduction

The breeding of new varieties in self-pollinating plants comprises three key features: the creation of variation by crossbreeding, stabilization by self-pollination or backcrossing over several generations, and the selection of the desired recombinants. At present, in rice breeding along with traditional breeding, biotechnological approaches are used, enabling to increase in the efficiency of the selection process. The most common approach for producing haploids and doubled haploids of rice is induced androgenesis in anther culture. In 1968, Niizeki and Oono were the first to report successful production of haploids from rice anther culture (Niizeki and Oono., 1968). This double-haploid biotechnology technique speeds up the breeding process by enabling the creation of stable plants within a single generation (Mishra and Rao, 2016; Lantos *et al.*, 2023). As a result, anther and microspore culture methods are commonly used in rice cultivation and the breeding of other crops to stabilize promising fissile plant populations (IRRI; Naik *et al.* 2017). Traditional breeding methods typically require 6-8 generations to produce homozygous lines (Grewal *et al.*, 2011), while haploid plant selection based on marker

traits is 5-6 times more efficient than selecting hybrid lines (Howes *et al.*, 1998; Pattnaik *et al.*, 2020). Haploid plants are highly valuable to breeders, as they facilitate the accelerated development of doubled haploid homozygous lines known as “pure lines” with all agronomically important genes fixed in a homozygous state (Datta, 2005). Nevertheless, the technique of culturing isolated anthers and microspores has not yet been widely adopted in practical breeding, largely due to the genotype-dependency of the regeneration process and the low success rate in producing green regenerant plants. Rice varieties, species, and subspecies exhibit varying degrees of responsiveness in anther culture: the highest responsiveness is found in glutinous rice, followed by the japonica subspecies, japonica/indica hybrids, indica/indica hybrids, and the indica subspecies (Medhabati *et al.*, 2009; Lantos *et al.*, 2023). There is also considerable variation in responsiveness within subspecies. Haploid plants offer several advantages in breeding efforts. Since haploids have only one set of chromosomes, breeders can directly observe mutations in the plants. When haploids undergo polyploidization, they become doubled haploids, which are completely homozygous. This absolute homozygosity allows breeders to stabilize valuable hybrid breeding lines and create “pure lines”. Utilizing rice doubled haploids enhances breeding efficiency and speeds up the development of genetically stable lines. The in vitro androgenesis technique allows for faster production of homozygous doubled haploids and facilitates the selection of lines with desirable traits, such as disease resistance, drought

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tolerance, cold tolerance, and improved grain quality. This method of producing doubled haploids is regarded as one of the most environmentally safe and harmless plant breeding methods, effectively addressing some of the limitations of traditional breeding methods. Therefore, the goal of this work is to produce doubled haploids in rice anther culture and select desirable lines for breeding.

2. Materials and Methods

The present work aimed to study *In-vitro* regeneration of plants through anther culture of rice. The objective was achieved by experimenting with the plant tissue culture in Laboratory at College of Biotechnology, Birsa Agricultural University, Ranchi. A thorough insight into the material used and the methodology applied during the course of the experimentation is as follows.

Plant materials-The Progeny of Imazethapyr herbicide tolerant segregants, grown in rice fields, BAU, Kanke, Ranchi were taken as experimental material for anther culture in this research.

Collection of explants-Panicles were selected based on the distance between the auricle of the flag and the penultimate leaves. Twenty booting panicles were selected when it was enclosed within the leaf sheath for each group (e.g., with distances of 5-7 cm, 7.1-9 cm and 9.1-13 cm) between the auricles of the leaves. Collection of the booting panicles was done in the morning around 8.30 AM. It was wrapped in aluminium foil and carried to the lab. The panicles were later sterilized and pre-treated before inoculation in callusing media.

Surface sterilization of the panicles (enclosed in leaf sheath) before cold treatment: Before cold treatment of the panicles (within leaf sheath), necessary sterilization was done to avoid infection in the anthers. The panicles were brought to the lab and washed thoroughly with tap water for 5 min. Then it was washed with distilled water. The panicles (within leaf sheath) were rinsed with 70% alcohol for 30 seconds and then again washed with distilled water. Sterilization with heat was also done by passing the panicles over the flame for a few seconds (avoid drying or burning).

Cold pre-treatment: The panicle after surface sterilization was wrapped in aluminium foil and kept at 4°C and 10°C for cold treatment for 7,9 and 15 days.

Preparation of media for inoculation of anthers and regeneration of the callus: Basal Callusing Media (CM) was prepared for inoculation of the cold-treated anthers after isolation. CM is a callus-inducing medium, which is a modified form of N₆ medium (Chu *et.al.*,1978). Later, after the callusing, it was put in regeneration media (PRM) for embryogenesis containing various hormonal regimes. For media preparation Na₂EDTA and Fe₂SO₄7H₂O are dissolved in hot water and stored in dark bottles, the hormones Naphthalene Acetic Acid (NAA) was dissolved in alcohol (absolute) and Benzyl-Adenine Purine (BAP) in NaOH (1N), half strength Murashige and Skoog (MS) basal medium was prepared by mixing one packet of commercially prepared mix (Hi media PT099) in 2 litres of double distilled water. The pH of the media is 5.6-5.8 to adjust the pH, 1N NaOH and 1N HCl were used.

Maintenance of culture: The culture bottles were kept in the culture room maintained at a temperature of 25±2°C in the dark and at 55-60% Relative Humidity (RH).

Surface sterilization of explant before inoculation: After the cold treatment, the enclosed panicles were taken out inside Laminar Air Flow (LAF) and it was cut open to expose the spikelets. The spikelets were surface sterilized with HgCl₂ (0.1%) for 10 min, and then washed 3-4 times with sterile water.

Isolation of anthers: The spikelets after sterilization were opened with the help of a scalpel to expose the anther. It was then isolated and directly inoculated into the Petri plate containing the callusing media, i.e. N₆(modified) media.

Plant regeneration: On reaching callus diameter greater than 3 mm, calluses were transferred to Murashige and Skoog media (MS media) (Murashige and Skoog, 1962), The highest plant regeneration was in PRM6 medium with a concentration of BAP (1.5 mg/L), NAA (1.0 mg/L) and kinetin (1.0 mg/L) pH maintained at 5.6-5.8. Nutrient medium was autoclaved at 121°C and 15 lbs per square inch pressure for 15 min. with added phytohormones. For plant regeneration, induced calli were subcultured to MS regeneration medium (Amirova *et al.*, 2022) and cultured under a 16 hour light and 8 hours dark photoperiod.

3. Results

The anthers were placed on the callus induction media, and the cultures were incubated in complete darkness. This was done to create an environment that the natural conditions that favour callus formation. The temperature during incubation was meticulously controlled and maintained at 25±2°C, which is within the optimal range for many plant tissue culture experiments. Incubating the anthers in the dark at this temperature allowed the cells to focus on proliferating into callus tissue without undergoing the differentiation that light exposure might encourage. The combination of the N₆ basal medium, supplemented with 2,4-D and NAA, along with the controlled dark and temperature conditions, provided an ideal environment for successful callus formation. In experiments, a combination of 2,4-D and NAA resulted in callus formation after 55 days from anthers taken from panicles with an auricle distance of 5-13 cm, specifically from the progeny of Imazethapyr herbicide-tolerant segregants.

It was observed that the time required for callus induction gradually increased for anthers from panicles with an auricle distance of 7.1-9 cm, while no callus formation was detected in 15 days cold treated anthers, but there was one callus formation in 7 days cold-treated anthers. Anthers from panicles with an auricle distance of 9.1-13 cm, after 9 days of cold treatment, were successfully induced to form callus formation. which was then followed by the regeneration of shootlets. The callus produced was characterized by a friable texture and a whitish colour. After callus formation quantity of callus was significantly increased by subculturing the calli every 30 days on the same medium.

To induce shoot regeneration in rice, clumps of anther-derived calli from the progeny of Imazethapyr-tolerant segregants were transferred to various plant regeneration media (PRM) like, PRM1, PRM2, PRM3, PRM4, PRM5, and PRM6. These media were based on Murashige and Skoog (MS media) and modified N₆ basal medium, a widely used plant tissue culture medium, but were enriched with different concentrations and combinations of auxins and cytokinins to promote shoot regeneration. Auxins and cytokinins are pivotal plant growth regulators that play complementary roles in tissue culture.

While auxins like Naphthaleneacetic acid (NAA) are important for root initiation and callus formation, cytokinins like kinetin and Benzyl amino purine (BAP) are crucial for stimulating cell division and shoot regeneration.

Table 1: Composition of Plant Regeneration Media (PRM)

S.N.	PRM 1		PRM 2		PRM 3	
	Component	Amount	Component	Amount	Component	Amount
1	CM	250 ml	CM	250 ml	1/2MS basal	250 ml
2	BAP	0.5 mg	BAP	0.75mg	NAA	0.25 mg
3	-	-	-	-	BAP	0.5 mg
4	-	-	-	-	-	-

S. N	PRM 4		PRM 5		PRM 6	
	Component	Amount	Component	Amount	Component	Amount
1	½ MS	250 ml	MS	1000ml	MS	1000ml
2	NAA	0.12gm	NAA	0.5 mg	Kinetin	1 mg
3	BAP	0.25 gm	BAP	0.5 mg	NAA	1 mg
4	-	-	-	-	BAP	1.5 mg

In this experiment poor regeneration was observed in PRM1, PRM2, PRM3, and PRM4, the anther calli transferred to PRM1, PRM2, PRM3, and PRM4 did not show any signs of successful shoot regeneration. Instead, blackening of the callus tissue was observed in these media, indicating a negative response to the hormonal composition. Blackening of callus is typically associated with tissue senescence, necrosis, which may result from an unsuitable balance of growth regulators or prolonged exposure to suboptimal culture conditions.

Table 2: Response of Callus in Plant Regenerated Media

MEDIA	Number of callus of clump	Response of callus	Albino (%)	Response days	Average response day
PRM 1	2	Black	-	15	15.00
	3	Black	-	15	
	2	Black	-	15	
PRM 2	1	Black	-	15	16.67
	1	Brown	-	18	
	2	Black	-	17	
PRM 3	2	Black	-	26	25.33
	3	Brown	-	25	
	2	No response	-	25	
PRM 4	3	No response	-	15	15
	4	Black	-	15	
	4	Black	-	15	
PRM 5	4	8 albino plant	100	20	21
	2	White	-	22	
	2	White	-	21	
PRM 6	3	5 albino plant	100	30	29.33
	2	Yellow white	-	30	
	4	7 albino plant	100	28	
C.D(0.05)			1.642		
SE(m)±			0.527		
C.V%			4.477		

However, after an initial period of poor response, the hormonal composition of the media was adjusted by increasing the concentrations of specific phytohormones, which resulted in the re-initiation of callus formation. This observation highlights the importance of precisely balancing auxins and cytokinins to maintain the viability of callus tissue and induce regeneration. Once the hormonal concentrations were adjusted, the calli were able to revert to a proliferative state, although no significant shoot regeneration was reported in these media.

In this experiment, a total of 20 plants were successfully regenerated from 11 distinct callus clumps derived from anther cultures. These callus clumps originated from the progeny of Imazethapyr herbicide-tolerant rice segregants, which had been subjected to tissue culture protocols aimed at inducing shoot regeneration through carefully optimised plant regeneration media (PRM). However, despite the successful initiation and development of plant shoots, all the regenerated plants exhibited albino plants.

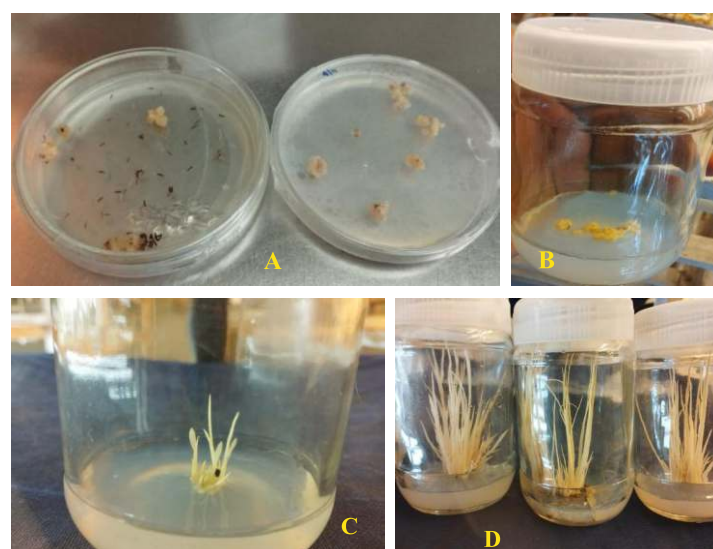


Figure 1; In vitro anther culture: (A) Callus induction from anther culture (B) Sub culturing of callus in transparent bottle (C) Initiation of regenerated plant from callus (D) Growth of regenerated albino plant

4. Discussion

In this experiment, explants were taken from progeny of Imazethapyr herbicide tolerant segregants, and their panicle were cold-treated for 7 days, 9 days, and 15 days at 4°C. Anthers from panicles with an auricle distance of 9.1-13 cm, after 9 days of cold treatment, were successfully induced to form callus formation. Which was then followed by the regeneration of shootlets. Specifically, cold pre-treatment at 8°C for 14 days has proven to be the most effective for anther culture in selected indica, japonica rice varieties, and inter-subspecific hybrids (Herath *et al.*, 2011), while a pre-treatment at 10°C for 7-9 days positively impacts indica rice hybrids (Mishra *et al.*, 2017). After callus formation six plant regeneration media are prepared i.e. PRM1, PRM2, PRM3, PRM4, PRM5, PRM6. Out of six media, only two media (PRM5 and PRM6) have successfully regenerated plants. In four media, only blackening of callus after an initial period was observed due to poor response; the hormonal composition of the media was adjusted by increasing the concentrations of specific phytohormones (NAA and BAP), which resulted in the re-initiation of callus formation. In this experiment, 20 plants were successfully regenerated from 11 different callus clumps, all of which were albino and therefore non-viable due to their lack of chlorophyll. The formation of albino plants likely resulted from a combination of hormonal imbalances, genetic factors, and the stress associated with the tissue culture process, all of which contributed to disruptions in chloroplast development. This outcome underscores the need for further optimization of tissue culture protocols to reduce albino plant formation and improve the efficiency of plant regeneration in androgenesis-based systems.

Therefore, the productivity and speed of generating consistent forms are greatly increased when advanced plant breeding is combined with traditional breeding approaches. The yield of green plants is commonly shown in many studies to be very low compared to albino plants in androgenesis *In-vitro* (Makowska and Oleszczuk, 2014). Albinism has been one of the unsolved challenges in anther and microspore culture *In-vitro* of cereals. Among the auxins, 2,4-dichlorophenoxyacetic acid (2,4-D) and naphthalene acetic acid (NAA) are the most commonly used for inducing callus in rice anther culture (Tapia *et al.*, 2002). Growth regulators, particularly auxins and cytokinins, are essential for regulating dedifferentiation in *In-vitro* cultures of crop plants. Adjusting the composition of the tissue culture medium, especially through the manipulation of plant growth regulators, can improve the success rate of the cultures (Mandal and Gupta, 1997). The combination of cytokinin and auxin in a 10:1 ratio has previously been shown to stimulate morphogenesis in plant tissue culture *In-vitro*, promoting massive plant regeneration (Amirova *et al.*, 2022). Furthermore, it is known that high concentrations of colchicine can lead to an increase in albino plant production. Colchicine has been noted to possess toxicity and does not always provide a positive outcome. (Hu and Liang, 1979).

5. Conclusion

The broad application of anther culture methods is constrained by the high incidence of chlorophyll-deficient regenerated plants, and ongoing research to refine techniques for double haploid breeding by anther culture. In this experiment, panicles were cold-treated for 9 days at 4°C and 10°C were successful in callus formation in N6 media. N6(modified) media was used for callus development and MS media was used for regenerated media supplemented with BAP, NAA, and kinetin.

Only callus blackening was observed in PRM1 to PRM4, while plant regeneration occurred in PRM5 and PRM6. This research involves 20 regenerated plants developed from 11 different clumps and all are albino plants.

6. Future scope

In-vitro androgenesis is an important tool in plant breeding for producing haploid and doubled haploid plants, which accelerates breeding, stabilizes traits, and improves the selection of recessive characters. Improving the efficiency of green haploid and doubled haploid regeneration in rice remains a key research priority. To reduce albino plant formation, future studies should focus on optimizing hormone balance in regeneration media, particularly by adjusting cytokinin levels and timing. Exploring genetic and epigenetic factors involved in chloroplast development may also help minimize albino phenotypes during androgenesis.

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No conflict of interest

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