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Standardization of *in-vitro* regeneration of *Oryza sativa* L.

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ABSTRACT

In the quest to enhance rice's agronomic traits at the cellular and molecular levels, biotechnological methods offer a more efficient and expeditious approach compared to conventional breeding techniques. In this study, we conducted in-vitro standardization and regeneration of Oryza sativa L. seeds using tissue culture techniques. We focused on exploring the morphogenetic response of seed and leaf explants cultured on media containing MS basal medium supplemented with various hormones, including BAP, Kinetin, Zeatin, and IAA in different combinations. The explants were derived from seven-day-old in vitro seedlings. Our investigation primarily centered on the induction of callus and the proliferation capacity of leaf and seed explants. The results revealed varying success rates in organogenesis between the two types of explants. Specifically, supplementation of MS medium with Zeatin (0.5 mg/l) and IAA (0.1 mg/l) led to 100% induction and proliferation of callus. These findings pave the way for the development of a rapid and highly effective genetic transformation strategy applicable for achieving diverse objectives, including CRISPR/Cas9 genome editing.

Keywords: *Oryza sativa L, explants, Callus induction, MS medium, Kinetin, BAP and IAA*

1. Introduction

Rice, serving as a cornerstone staple food crop globally, predominantly thrives in the fields of Asia, yet its significance is overshadowed by its vulnerability to a myriad of pests and diseases, culminating in substantial losses in both yield and quality [1-2]. The imperative for a dependable *in vitro* regeneration system resonates profoundly in the realm of genetic modifications aimed at enhancing economically pivotal fragrant rice varieties [3]. Conventional breeding methods, though earnestly pursued, have often fallen short in augmenting rice yields to meet escalating demands. In the landscape of biotechnology, the cultivation of rice plants *in vitro* from cellular or tissue explants emerges as a pivotal strategy for crop genetic enhancement. The research underscores the pivotal role of exogenously applied plant growth regulators, including naphthalene acetic acid (NAA), benzylaminopurine (BAP), and thidiazuron (TDZ), in conjunction with kinetin (Kin), in significantly bolstering the frequency of rice regeneration [4]. However, the successful genetic improvement of rice hinges upon the identification of genotypes conducive to callus growth and proficient in vitro plant regeneration.

Noteworthy are the multifaceted factors influencing callus induction and regeneration potential, extending beyond gene and explant types to encompass culture conditions and medium composition, including the judicious integration of plant growth regulators [5]. Thus, the pursuit of optimal conditions for callus induction and subsequent regeneration represents a nuanced endeavor, demanding meticulous attention to diverse variables within the tissue culture environment. This synthesis underscores the intricate interplay of factors shaping rice biotechnology and highlights the imperative for comprehensive strategies to navigate the complexities of in vitro regeneration, ultimately steering towards the realization of enhanced rice varieties capable of withstanding the rigors of contemporary agricultural landscapes.

2. Materials and methods

2.1. Plant material

The variety rice (*Oryza sativa*) was chosen for the current study. The Agricultural Research Institute (ARI) in Rajendranagar, Hyderabad, furnished the seeds.

2.2. Preparation of culture media

In our investigation, Murashige and Skoog's (1962) classic culture medium laid the groundwork for the production of multiple shoots, callus induction, and overall plant regeneration. The composition of the medium is delineated in the tables below, encompassing a meticulous integration of growth hormones, carbon sources, and precise quantities of inorganic and organic nutrients. The remaining volume was supplemented with double-distilled water to achieve optimal conditions.

Prior to the autoclaving process, pH adjustments were conducted to attain a pH of 5.8 using either 1N NaOH or 1N HCl at 1.06 Kg/cm3 (15 psi) and 121°C for 15 minutes. This stringent pH control ensures the stability and efficacy of the culture medium, providing an environment conducive to the growth and development of plant tissues under investigation.

2.3. Preparation of stock solutions

The following stock solutions were prepared and stored in a refrigerator for subsequent use in media preparation. Each stock solution was meticulously prepared to maintain its stability and integrity, ensuring consistent and reliable results during media preparation and subsequent experiments. Refrigeration helps to prolong the shelf life of the solutions and prevents degradation of their components over time.

Stock: Major stock 1

Components	g/500ml
KNO_3	38.0 gm
NH_4NO_3	33.0gm
KH_2PO_4	3.4gm
$MgSO_4.7H_2o$	7.4gm

The aforementioned salts were weighed and their respective amounts dissolved in approximately 300 milliliters of distilled water while being continuously stirred. Distilled water was added to the final capacity to make it reach 500 ml. The reagent bottle was labeled as MS-I with date of preparation and stored at 4-8°C

Stock: Major stock 2

Component	g/250ml
$CaCl_2.2H_2o$	11.0 gm

After dissolving eleven grams of CaCl2.2H2O in roughly 100 milliliters of distilled water, the final volume was increased to 250 milliliters using distilled water. The preparation date was written on the reagent bottle labeled MS-II, which was kept between 4 and 8°C.

Stock Minor stock

Components	Milligrams/250ml
$MnSO_4.4H_2O$	422.5mg
$ZnSO_4.7H_2O$	215.0mg
H_3PO_3	155.0mg
KI	20.0mg
$Na_2MoO_4.2H_2O$	625mg
$CuSO_2.5H_2O$	0.63mg
$CoCl_2.6H_2O$	0.63mg

After weighing and dissolving the designated amounts of the aforementioned salts in roughly 150 ml of distilled water, 250 ml of distilled water was added to complete the volume. The reagent container was kept at 4–8 degrees Celsius and marked MS-III along with the preparation date.

Stock: Iron stock

Components	Grams/100ml
Na_2-EDTA	1.835gm
$FeSO_4$	1.390gm

A glass beaker covered in brown paper containing approximately 300 milliliters of distilled water was heated using a magnetic stirrer and heater. Boiling water was used to dissolve 1.835 g of Na_2EDTA and 1.390 g of $FeSO_4.7H_2O$ while stirring continuously. The finished amount was transferred into an amber (brown) reagent container and filled to a capacity of 500 ml using distilled water. With a preparation date and an MS-IV label, the reagent bottle was kept between 4 and 8 degrees Celsius.

Stock: Vitamins

Components	Milligrams/500ml
Nicotinic acid (Niacin)	20mg
Pyridoxine HCl	20mg
Thiamine HCl	20mg

The designated amounts of these vitamins were weighed, and they were each dissolved in roughly 50 milliliters of distilled water before adding more distilled water to get the final level of 100 milliliters. Reagent bottle B5, prepared date on label, kept between 4 and 8 degrees Celsius.

2.4. Preparation of hormone stock solutions

In order to induce plant regeneration from various explants, we augmented the MSB5 basal medium with a variety of growth regulators, each in different combinations and concentrations. The plant growth regulators employed in this study included BAP (6-Benzyl aminopurine) and 2,4-D (2,4-dichlorophenoxyacetic acid). The stocks of these growth regulators were prepared according to the following procedure: Initially, hormones were dissolved in specific organic solvents, after which the final volume was adjusted using distilled water.

Hormone	Solvent	Final vol. with
BAP	1N NaOH	Distilled Water
2,4-D	1N C_2H_5OH 1N NaOH	Distilled Water

Weighed 10 mg of each hormone in a 1.5 ml eppendorf tube and dissolved in few drops of specific organic solvent as indicated in the above table. After complete dissolution of the hormone, it was transferred into a 15 ml screw cap tube and the final volume was made up to 10 ml with distilled water to get 1mg/ml final concentration. The stocks solution of hormones was filter sterilized using disposable sterile syringe filter (0.22µm thickness) within the laminar air flow. Hormone stocks were labeled and stored at 4-8°C.

2.5. Media preparation

Stock	Quantity/Liter
Major stock	100 ml
Minor stock	10 ml
Iron stock	10 ml
Vitamins	5 ml
Sucrose	30 gm (3%)
Agar agar	8 gm (0.8%)

2.6. Surface sterilization

The seeds underwent a thorough sterilization process outlined. Initially, the seeds were washed with sterile distilled water to eliminate any external debris or contaminants present on the surface. Under a laminar flow cabinet, the seeds were sterilized using 70% ethanol for 90 seconds to eradicate surface contaminants effectively. Subsequently, the seeds were immersed in a 0.1% $HgCl_2$ (mercuric chloride) solution for a duration of 2 minutes to ensure thorough sterilization. Following the sterilization steps, the seeds underwent extensive rinsing with sterile distilled water 2-3 times to eliminate any residual ethanol or mercuric chloride solution, thereby ensuring the seeds were devoid of contaminants before further experimentation.

2.7. Germination of seeds

The germination of rice seeds was conducted to achieve high frequency and uniform seed germination. Following surface sterilization, ten seeds were directly placed onto MS basal medium supplemented with 0.8% agar in culture bottles. The bottles were then incubated for 7 days at a temperature of 25°C ± 2 under a 16-hour photoperiod. Data regarding seed germination were recorded after the 7-day incubation period.

After the initial week, fully grown healthy shoots were selected for further transformation experiments. The conditions for in vitro culture incubation were maintained at 25°C with a light intensity of 4000 Lux and a photoperiod of 16 hours, along with 70% humidity. The impact of the basal medium on seed germination was assessed as part of the study.

2.8. In vitro induction of callus
The purpose of the trials was to investigate the induction of callus formation *in vitro.*

2.8.1. Preparation of culture media
[6-7] classic culture medium served as the foundation for investigating callus induction, multiple shoot formation, and plant regeneration in its entirety. Table 2 outlines the composition of the medium, including growth hormones, carbon sources, and precise quantities of inorganic and organic nutrients. The remaining volume was supplemented with double-distilled water. Prior to the autoclaving process, pH adjustments were made to achieve a pH range of 5.6–5.8 using either 1N NaOH or 1N HCl at a pressure of 1.06 Kg/cm3 (15 psi) and a temperature of 121°C for 15 minutes.

2.9. Influence of BAP on callus induction
The seeds were placed onto MS basal medium supplemented with varying concentrations of BAP (0.1, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, and 5.0 mg/L), along with 3% sucrose and 0.8% agar. These cultures were carefully maintained at a consistent temperature of 25±2°C under a 16-hour photoperiod. To facilitate photosynthesis, a photosynthetic flux density (PPFD) of 83.6 µEm^-2S^-1 was provided by white fluorescent tubes. This controlled environment was essential to promote optimal growth conditions for the seeds and subsequent plant development.

2.10. Influence of BAP, KN, ZN, NAA and IAA on seed and leaf
The explants, comprising seeds and leaves, were placed onto MS media supplemented with varying concentrations of several hormones to induce callus formation. These concentrations included: 1 mg/L BAP, 0.1 mg/L NAA; 1 mg/L BAP, 0.2 mg/L NAA; 1 mg/L BAP, 0.1 mg/L IAA; 2 mg/L BAP, 0.1 mg/L IAA; 3 mg/L BAP, 0.1 mg/L IAA; 1 mg/L Kin; 2 mg/L Kin; 0.5 mg/L Zeatin, 0.1 mg/L IAA; 0.5 mg/L Zeatin, 0.5 mg/L IAA; 1.0 mg/L Zeatin, 0.5 mg/L IAA; 1.0 mg/L Zeatin, 1.0 mg/L IAA. Subsequent to every seven days, subculturing of the explants was carried out. Upon observation of callus growth, the explants were transferred to shoot initiation media supplemented with 0.2 mg/L BAP. The cultures were meticulously maintained under a 16-hour photoperiod with a photosynthetic flux density (PPFD) of 83.6 µEm^-2S^-1 provided by white fluorescent tubes. A temperature range of 25±2°C was upheld to ensure optimal conditions for sustained culture growth.

Results
Over the past few decades, significant efforts have been dedicated to developing rapid regeneration methods and achieving high-frequency induction of transformants in rice. Despite the success of various protocols developed for different cultivars in different regions, a suitable protocol for one of the most significant types, RP Bio-226, remains elusive. Our investigations have aimed to address this gap, and the following outcomes are the result of the experiments conducted in this direction.

2.11. Effect of basal medium on callus induction
In order to induce seed germination in RP Bio-226, solid MS basal medium formulations containing sucrose were tested. For the purpose of germination and callus induction, surface-sterilized seeds were stored in the dark for six days, followed by a 16-hour photoperiod in the light. Within seven days, the solid MS basal media began to germinate out of all the basal media. The highest percentage of seed germination was supported by solid MS medium containing sucrose.

Fig. 1: Seed germination of Rice on MS basal medium

2.12. Effect of BAP on callus induction
The previous reports on rice served as the basis for the selection of MS basal medium in the current study. The seeds cultivated on MS basal medium supplemented with BAP (0.5-2.0 mg/l) exhibited varying degrees of responsiveness in the induction of callus, contingent on the tested concentration. The incidence of callus induction in seeds was examined at BAP concentrations, with a high of 100% in 0.4 mg/l BAP.

Fig 2. : Seed on MS medium with BAP 0.4 mg/lt.

2.13. Impact of BAP, KN, ZN, and IAA on Seed and Leaf for Callus Induction
The morphogenetic response of seed and leaf explants cultured on MS basal medium supplemented with a variety of hormones, including BAP, Kinetin, Zeatin, and IAA in different combinations, exhibited significant variation. Explants were derived from seven-day-old *in vitro* seedlings. Compared to Kinetin (50–60%) and Zeatin+IAA (35–85%), seeds demonstrated remarkably favorable responses in MS basal medium supplemented with BAP and IAA, resulting in the highest regeneration frequency (>90%). Among the tested combinations, Zeatin 1.0 mg/l + IAA 0.5 mg/l (7.36) yielded the maximum number of calluses, with statistically insignificant differences observed among Zeatin 0.5 mg/l + IAA 0.5 mg/l (7.10) and Zeatin 1.0 mg/l + IAA 1.0 mg/l (5.76). Notably, Zeatin 0.5 mg/l + IAA 0.1 mg/l produced the highest quantity of callus per explant. Additionally, the medium supplemented with BAP 0.4 mg/l + IAA 0.1 mg/l (8.06) demonstrated the second-best response in terms of callus/explant quantity.

Furthermore, the leaf exhibited exceptional responsiveness to BAP plus IAA, displaying the highest regeneration frequency (>90%) compared to Kinetin (40–60%) and BAP+IAA (25–75%). Among the combinations tested,

Zeatin 0.5 mg/l + IAA 0.1 mg/l (5.89 shoots/explant) induced the most callus, followed closely by Zeatin 0.5 mg/l + IAA 0.5 mg/l (5.32) and Zeatin 1.0 mg/l + IAA 0.5 mg/l (4.09). In terms of callus/explant quantity, the medium supplemented with BAP 2.0 mg/l + IAA 0.1 mg/l (5.87) exhibited the second-best response, followed by Kinetin 2 mg/l (2.01).

These findings underscore the pivotal role of hormone combinations and concentrations in influencing callus induction and regeneration potential in both seed and leaf explants, offering valuable insights for optimizing tissue culture protocols in rice breeding programs.

Fig. 3. Effect of BAP and IAA, on callus induction from seed and leaf.

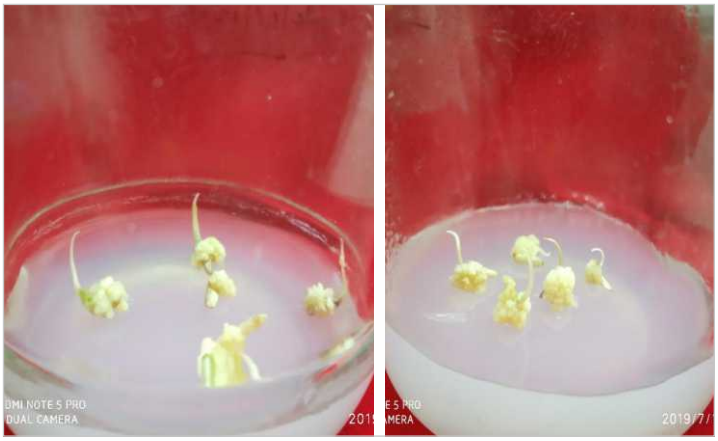

Fig. 4. Effect of Zeatin 0.5 mg/l + IAA 0.1 mg/l, on callus induction from seed and leaf

3. Discussion and Conclusion

Rice (*Oryza sativa* L.), a member of the Poaceae family and sub-family Oryzoidea, holds a pivotal role as a staple food for 70% of the global population. Its cultivation encompasses one-fifth of the land dedicated to cereal crops, emphasizing its profound significance in global agriculture [8-10]. Rice provides 20% of the world's daily calories, primarily consumed as a whole grain. With thousands of rice varieties developed to adapt to diverse climatic conditions, the grain boasts a spectrum of qualitative attributes related to appearance, cooking, eating, and product development [11-12]. Both traditional and modern plant breeding tools play pivotal roles in rice improvement. In the past few decades, advancements in transgenic technology have revolutionized crop breeding. Initially demonstrated with the genetically modified tomato variety [13-14], transgenic technology has made significant strides, overcoming barriers to hybridization. Currently, approximately 102 million hectares of land across 22 nations are cultivated with genetically modified crops, spanning 25 different varieties. These advancements owe much to progress in genetics, molecular biology, plant physiology, entomology, plant pathology, plant tissue culture, and recombinant DNA technology [15]. The investigation focused on inducing callus and assessing the proliferative capacity of leaf and seed explants. Variances were noted in the success rates of organogenesis between the two types of explants. Notably, the addition of Zeatin (0.5 mg/l) and IAA (0.1 mg/l) to the MS medium resulted in 100% callus induction and proliferation. This development signifies the emergence of a rapid and highly effective genetic transformation strategy, offering promising applications such as CRISPR/Cas9 genome editing. These advancements represent a significant leap forward in rice biotechnology, unlocking new pathways for crop improvement and addressing global food security challenges. As research advances, the integration of cutting-edge technologies holds the potential to revolutionize rice production, ushering in enhanced yield, resilience, and nutritional quality.

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