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A Robust *In Vitro* Regeneration Protocol for *Oryza sativa* MTU 1010 as a Gateway to Genetic Transformation

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ABSTRACT

Developing a dependable, efficient and in vitro regeneration system is crucial for facilitating genetic transformation in rice, particularly for indica cultivars like MTU 1010, which are often resistant to tissue culture techniques. This study presents an optimized protocol that significantly improves regeneration efficiency in Oryza sativa cv. MTU 1010. Mature seeds dehusked, surface-sterilized, and cultured on Murashige and Skoog (MS) medium augmented at 2.5 mg/L Utilization of the 2,4-dichlorophenoxyacetic acid (2,4-D) at a concentration of 30 g/L maltose to induce callus development. Embryogenic calli, predominantly originating from the scutellum, had favorable morphological characteristics, including compactness and friability, within one week of incubation. Among the various treatments, a concentration of 2 mg/L 2,4-D produced the highest callus induction rate of 87.89%. And MS medium supplemented at 0.5 mg/L α-naphthaleneacetic acid (NAA) and 2 mg/L 6-benzylaminopurine (BAP) is used for shoot organogenesis. supported optimal regeneration, achieving an efficiency of 87.5%. Regenerated shoots exhibited healthy elongation and rooting, with a survival rate of 90% upon acclimatization. This reproducible protocol addresses cultivar-specific challenges and provides a valuable framework for future genetic engineering efforts, including Agrobacterium-mediated gene transfer and CRISPR/Cas9 applications aimed at improving stress tolerance and yield potential in rice.

Keywords: *Oryza sativa MTU 1010; in vitro regeneration; embryogenic callus; shoot induction; Agrobacterium transformation.*

1. Introduction

Rice is the most important cereal crops in the world and is the main food supply for around half of the world's people [1]. Asia produces and consumes about 90% of global rice [2]. Among Asian countries, China, India, Indonesia, Bangladesh, Japan, and Vietnam contribute nearly 80% of rice production and consumption. The major Asian countries account for nearly 80% of the production and consumption of rice, and the India holds the 2nd position of the world's largest producer of rice, making a substantial contribution to the worldwide market rice output [3]. In South and Southeast Asia, including India and Bangladesh, rice is cultivated extensively across diverse agro-ecological zones. In India and Bangladesh, rice is grown around the year during Aman, Aus, and Boro seasons in different climatic zones, and serves as farmers' livelihoods, food, and nutritional security [4]. Despite the crop's significance, rice production is threatened by extremes of climatic conditions driven by various abiotic and biotic factors [5]. These extreme climate conditions significantly reduce the acreage of cultivation and yield in rice [6]. This highlights the critical necessity for breeding rice cultivars with improved resistance to both abiotic and biotic stresses, alongside increased yield potential, to sustain the food requirements of an expanding global population. Recent progress in plant biotechnology has established genetic transformation as a potent strategy for crop enhancement, particularly for boosting yield and developing climate-resilient varieties.

Despite this potential, *indica* rice cultivars such as MTU 1010 remain highly resistant to in vitro tissue culture and regeneration, presenting a significant challenge to efficient genetic transformation. Genotype dependency, Low regeneration, and transformation frequency in indica rice slow down the pace of its genetic improvement for key traits utilizing genome engineering technologies [7]. A multitude of studies has been conducted on diverse medium types, hormonal combinations, inorganic component compositions, and solidifying agents for the induction and regeneration of rice callus [8-10]. But the previous rice regeneration and transformation protocol is not efficient and possesses some bottlenecks. Therefore, the development of a stable and consistently effective The in vitro regeneration methodology is crucial for enabling effective genetic engineering endeavors. In this research, we established a high-frequency regeneration protocol for *O. sativa* (MTU 1010), providing a reliable platform for future *Agrobacterium*-mediated transformation to address the pressing challenges in rice production.

2. Materials and Methods

2.1 Plant Material

The Mature, dehusked seeds of *Oryza sativa* cv. MTU 1010 underwent surface sterilization to eradicate microbiological contaminants. The sterilization process consisted of an initial exposure to 70% (v/v) ethanol for 1 minute, succeeded by immersion in a 4% sodium hypochlorite (NaOCl) solution augmented with 2–3 drops of Tween-20 for 10 minutes. to enhance surface penetration. To further prevent fungal growth, the seeds were treated with a 0.5% (w/v) Bavistin solution for 5 minutes, as per [11]. Each step was followed by five successive rinses with sterile distilled water to ensure complete removal of sterilizing agents. The disinfected seeds were then air-dried on autoclaved filter paper under sterile conditions before being transferred onto the callus induction medium in 90-mm sterile Petri plates for culture initiation.

Media Preparation

Murashige and Skoog (MS) basal medium of (4.4 g/L) was augmented at the 2,4-dichlorophenoxyacetic acid (2,4-D), 30 g/L maltose, and 4 g/L Gelrite for callus induction. The co-cultivation medium was formulated with half-strength MS salts (2.2 g/L), supplemented with 15 g/L sucrose, 10 g/L glucose, and 4 g/L Gelrite. Following autoclaving at 121°C for 20 minutes, 100 µM acetosyringone was incorporated into the medium chilled to 60–70°C. The selection medium consisted of full-strength MS supplemented with 2 mg/L BAP, 2.5 mg/L 2,4-D, 1 mg/L NAA, 30 g/L maltose, 1 g/L proline, 0.3 g/L casein hydrolysate, 100 mg/L sorbitol, and 4 g/L Gelrite, with the pH adjusted to 5.2 prior to sterilization. The regeneration medium resembled the selection medium, substituting maltose with sucrose (30 g/L), and its pH was calibrated to 5.8. All media were autoclaved at 121°C for 20 minutes and thereafter distributed under sterile conditions into suitable culture containers.

2.2 Callus Induction, Proliferation, and Regeneration

Surface-sterilized mature seeds of *Oryza sativa* L. cv. MTU 1010 were cultured on Murashige and Skoog (MS) basal medium (4.4 g L^{-1}) supplemented with 2.5 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), 30 g L^{-1} maltose, and 4 g L^{-1} Gelrite for callus induction. The pH of the medium was adjusted to 5.8 before autoclaving. Cultures were incubated in complete darkness at 25 ± 2°C for 3–4 weeks to facilitate the formation of embryogenic calli. Compact, nodular, and actively proliferating embryogenic calli (Figure 1; stages 2–3) were selected and transferred to regeneration medium containing MS salts supplemented with 2.0 mg L^{-1} 6-benzylaminopurine (BAP), 0.5 mg L^{-1} α-naphthaleneacetic acid (NAA), and 30 g L^{-1} sucrose. The cultures were maintained under a 16 h light/8 h dark photoperiod at 25 ± 2°C with a light intensity of approximately 50 µmol m^{-2} s^{-1}. Shoot regeneration and plantlet development were monitored over a period of 4–6 weeks (Figure 1; stages 4–5). Regeneration efficiency was assessed by recording the percentage of *calli* producing shoots, the average number of shoots regenerated per callus, and the rooting frequency of regenerated plantlets. Successfully regenerated plantlets were subsequently transferred for further growth and acclimatization.

2.3 Acclimatization of Regenerated Plantlets

Well-rooted regenerated shoots (Figure 1, stage 6) were delicately removed from the culture jars and meticulously cleaned with sterile distilled water to eliminate any remaining culture material. The plantlets were subsequently transplanted into pots filled with a sterilized blend of soil, sand, and compost in equal ratios (1:1:1). The pots were maintained in a controlled environment chamber with regulated temperature and humidity to ensure successful acclimatization. The young plants underwent a gradual hardening process over a period of days to allow them to adjust to ex vitro conditions.

Following acclimatization, the plantlets were transferred to greenhouse conditions, where they continued to grow and establish under natural light and ambient environmental settings.

2.5 Data Acquisition and Statistical Evaluation

Callus induction percentage was assessed following a four-week incubation period on the callus induction medium. Similarly, shoot regeneration efficiency and the mean number of shoots per callus were documented after four weeks of cultivation on the regeneration medium. Rooting efficacy, encompassing the proportion of root induction and the average quantity of roots per regenerated plantlet, was assessed following four weeks of growth under rooting conditions.

2.6 The frequency of callus induction (%) was determined using the following formula

$$\text{Callus induction (\%)} = \frac{\text{Number of explants producing calli}}{\text{Total number of explants cultured}} X100$$

Shoot regeneration frequency (%) was determined as:

$$\text{Shoot regeneration (\%)} = \frac{\text{Number of calli regenerating shoots}}{\text{Total number of calli transferred for regeneration}} X100$$

Root induction frequency (%) was calculated as:

$$\text{Root induction (\%)} = \frac{\text{Number of plantlets with roots}}{\text{Total number of plantlets transferred for rooting}} X100$$

All tests were conducted used within the Completely Randomized Design (CRD), and the data underwent analysis of (ANOVA) and to evaluate the significance of changes between treatments. Duncan's Multiple Range Test (DMRT) were also conducted at 5 % significance level ($p < 0.05$) to differentiate the means and ascertain statistical significance.

3. Results

3.1 Callus Induction and Morphology

Within three weeks of culture, mature dehusked seeds of *Oryza sativa* cv. MTU 1010 produced embryogenic calli when grown on MS medium fortified with 2.5 mg/L 2,4-D and 30 g/L maltose. Callus formation was initiated from the scutellum region within one week of incubation under dark conditions. The developing calli were compact, nodular, and exhibited a light yellowish to cream color, characteristic of embryogenic tissue suitable for subsequent transformation procedures (Figure 1, stages 2–3). After three weeks, calli became more globular and friable in texture (Figure 2b), which is a desirable feature for *Agrobacterium*-mediated infection and T-DNA delivery.

Callus induction efficiency varied among treatments, with induction rates ranging from 70.55% to 87.89%. The application of 2 mg/L of 2,4-D alone produced the greatest callus induction. (87.89%) in MTU 1010 when cultured in the dark. This response aligns with findings in the Sadamota cultivar, where 100% callus formation was achieved using 2 mg/L 2,4-D, followed by a 95.51% Induction rate utilizing a mixture of 3 mg/L 2,4-D and 10 mg/L kinetin. These observations highlight the necessity of genotype-specific adjustment of auxin concentrations and confirm the efficacy of 2,4-D in facilitating embryogenic callus development in indica rice cultivars.

3.2 Shoot Regeneration and Plantlet Recovery

When subcultured on to regeneration medium containing MS salts added with 2 mg/L 6-benzylaminopurine (BAP) and 0.5 mg/L NAA, embryogenic calli exhibited vigorous shoot development under a 16/8 h light/dark cycle. The highest regeneration frequency recorded in MTU 1010 was 87.5% (Figure 1, stages 4–5). Regenerated shoots exhibited healthy elongation and greening, with subsequent rooting achieved efficiently on hormone-free MS medium (Basal MS medium). Rooted plantlets were acclimatized in a controlled growth chamber, achieving a high survival rate of 90% upon transfer to greenhouse conditions (Figure 1, stage 6).

3.3 Potential for Genetic Transformation

The friable texture and high regenerative potential of the calli make them highly amenable to *Agrobacterium* infection, enhancing the likelihood of successful T-DNA integration and recovery of transgenic plants. The combination of high callus induction and shoot regeneration frequencies in MTU 1010 supports the utility of this protocol as a foundation for genetic transformation studies targeting stress tolerance and yield enhancement.

4. Discussion

The development of an efficient and genotype-independent regeneration system remains a significant bottleneck in indica rice transformation efforts. Our study demonstrates a robust and reproducible in vitro regeneration protocol for MTU 1010, achieving callus induction frequencies up to 87.89% and shoot regeneration frequencies of 87.5%. These values surpass those reported in previous studies for other indica cultivars, which often recorded lower regeneration efficiencies due to genotype recalcitrance [12-13]. Callus formation initiated from the scutellum region within a week of culture, consistent with findings in other indica varieties where scutellum-derived calli exhibited higher regenerative potential [14]. The use of 2.5 mg/L 2,4-D as the primary auxin was critical in inducing embryogenic calli with desirable morphological characteristics. Interestingly, in comparative studies, Sadamota displayed a maximum callus induction frequency (100%) under 2 mg/L 2,4-D, indicating possible genotypic variation in auxin sensitivity. The high shoot regeneration efficiency observed on cytokinin-rich medium (BAP + NAA) aligns with the hormonal requirement for shoot organogenesis in rice tissue culture. The successful acclimatization of regenerated plantlets with a 90% survival rate further underscores the practical utility of the protocol, this study addresses the genotype-specific challenges in indica rice regeneration by optimizing hormonal combinations and culture conditions.

5. Conclusion and Future Directions

This study successfully establishes a robust and reproducible in vitro regeneration protocol for *Oryza sativa* cv. MTU 1010, a widely cultivated indica rice variety. The protocol demonstrated high callus induction frequencies (up to 87.89%) and efficient shoot regeneration (87.5%), with a remarkable plantlet acclimatization survival rate of 90%. The friable and embryogenic nature of the induced calli, combined with the optimized hormonal regime, positions this system as a reliable platform for downstream genetic transformation applications. The genotype-specific challenges often associated with indica rice tissue culture were effectively addressed through precise adjustments in auxin and cytokinin concentrations, highlighting the adaptability of this protocol across related cultivars. Such advancements can accelerate functional genomics studies and trait improvement programs, particularly for stress tolerance and yield enhancement in rice. Looking ahead, this regeneration system offers promising potential for integration with *Agrobacterium*-mediated transformation and CRISPR/Cas9-based genome editing techniques. Future research could focus on evaluating transformation efficiencies using this protocol, optimizing selectable marker systems, and applying it to introduce genes conferring resistance to abiotic stresses such as submergence and salinity. Additionally, extending this approach to other recalcitrant indica cultivars may broaden its utility in rice improvement initiatives.

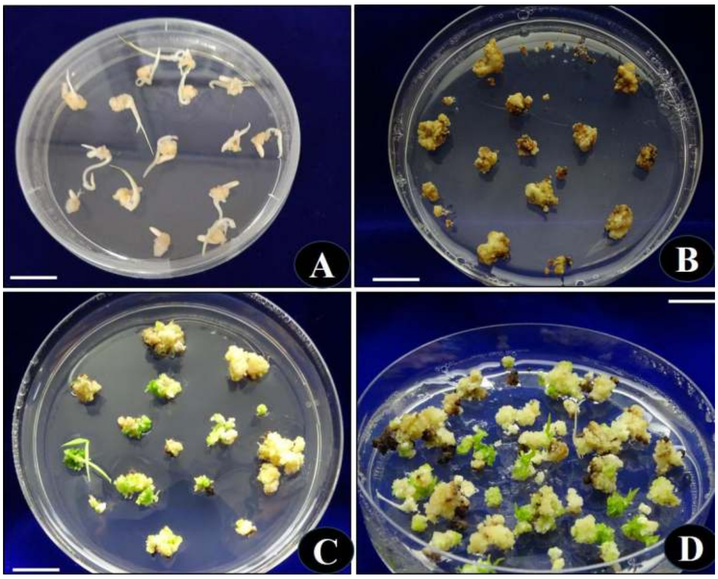

Fig. 1. In vitro callus induction and plant regeneration in Oryza sativa L. cv. MTU1010.

A. Callus initiation from immature embryos on Callus Induction Medium (CIM) containing MS salts (4.4 g/L), maltose (30 g/L), and 2,4-D (2.5 mg/L).
B. Proliferation of embryogenic calli on CIM.
C. Greening and shoot primordia emergence on Selection Medium supplemented with BAP (2 mg/L) and NAA (1 mg/L).
D. Multiple shoot regeneration on Regeneration Medium with BAP (2 mg/L), NAA (1 mg/L), and Kn (1 mg/L).

Scale bars = 1 cm.

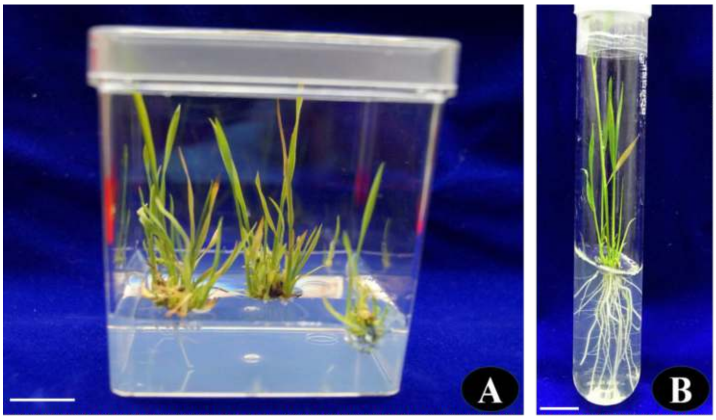

Fig. 2. In vitro rooting and plantlet development of Oryza sativa L. cv. MTU1010.

A. Regenerated shoots transferred to rooting medium containing half-strength MS salts (2.2 g/L) and sucrose (15 g/L) showing robust shoot proliferation.
B. Well-developed roots and shoots in test tubes after culture on rooting medium.

Scale bars = 1 cm.

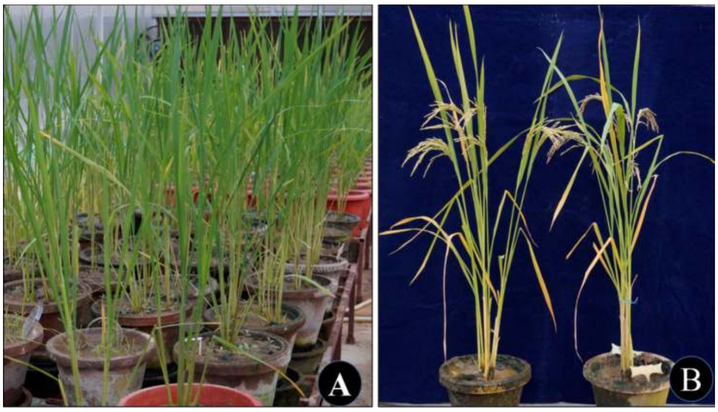

Fig. 3. Acclimatization and maturity of regenerated rice plants (Oryza sativa L. cv. MTU1010).

A. Hardened plantlets successfully transferred to soil and grown under greenhouse conditions.
B. Mature rice plants at the reproductive stage showing normal panicle development after acclimatization.

Author contributions

AKV, VS, and MRD were responsible for the conceptualization and design of the study. Experimental work was carried out by AKV and VS. The initial manuscript draft was prepared by AKV, SKD, and MKB. VS, SKL, and MRD provided critical review and contributed to manuscript revisions. All authors reviewed and approved the final manuscript prior to submission.

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Conflict of interest

The authors declare no conflicts of interest.

Data Availability Statement

The manuscript has no associated data, or the data will not be deposited.

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