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The Effect of Basal Medium on Callus Induction and Plant Regeneration in Anther Culture of Rice (*Oryza sativa* L.)

Yoanita Fadlilah Iriani[1,2*], Dian Catur Prayantini[2], Hartinio Natalia Nahampun[2], Retno Mastuti[1], Nunung Harijati[1]

[1] Department of Biology, Faculty of Mathematics and Natural Sciences, Brawijaya University, Malang, East Java, Indonesia.
[2] Tissue Culture Laboratory, Department of Biotechnology, PT. BISI International, Tbk., Kediri, East Java, Indonesia.

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Corresponding Author:
Yoanita Fadlilah Iriani
yoanita204@gmail.com

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Abstract: Anther culture is an effective tissue culture technique for rapidly producing doubled haploid (DH) plants, significantly shortening the breeding cycle by 2-3 years compared to conventional methods. This study aimed to evaluate the effects of callus induction basal medium (B5 Gamborg and N6 Chu) and subsequent plant regeneration in both Japonica and Indica rice genotypes. The experiment was conducted using a completely randomized design (CRD) with two treatment factors. The first factor was genotype (G), comprising Nipponbare (G1), RCKJ 05 (G2), RCKJ 10 (G3), RCKJ 15 (G4), RCKJ 25 (G5). The second factor was the basal medium (M), consisting of N6 Chu medium (M1) and B5 Gamborg medium (M2). Each treatment combination (genotype x medium) was replicated three times, with one Petri dish (containing 125 anthers) per replicate. Parameters observed included callus induction frequency (CIF), green plantlet regeneration (RGP), and albino plantlet regeneration (RAP). The results demonstrated that both genotype and the basal medium for callus induction significantly influenced callus induction frequency and green plantlet regeneration.

Keywords: B5 Gamborg; Callus; Double haploid; N6 Chu; Rice

Introduction

Rice (*Oryza sativa* L.) is a staple food in Indonesia, crucial for national food security. Anther culture, a tissue culture technique, significantly accelerates the development of new plant varieties, including rice (Kalinina & Kostylev, 2023; Lantos et al., 2023). This biotechnology method is highly effective in rapidly generating doubled haploid (DH) plants, which can shorten the breeding cycle by 2-3 years compared to conventional approaches (Das et al., 2022).

Rice anthers culture use microspore-containing anthers from young panicles as explants for *in vitro* cultivation (Cimò et al., 2017; Orłowska et al., 2020). These uninucleate microspores are isolated and cultured on specialized media to induce callus formation, then can be regenerated into fertile green plants (Nurhasanah et al., 2016).

The success of anther culture is influenced by a multiple factors, including genotype, composition of the culture media, cold pre-treatment, microspore phase, and environmental conditions of the donor plant (Chen et al., 2022; Haridhi, 2023; Sivachandran et al., 2017; Yildiz, 2024). Genotype and media composition have been identified as the primary factors that influence the response of rice anther culture (Kaushal et al., 2014). This was due to the direct impact of these factors on the ability of microspores to induce callus and subsequently regenerate green plants (Reddy et al., 1985).

The success of anther culture is determined by plant genotypes, genetic factors regulate cell totipotency, the activity of genes that control cell division and

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differentiation, and sensitivity to growth regulators in the culture medium (Long et al., 2022; Duyi et al., 2017). A multitude of studies have demonstrated that the japonica subspecies of rice exhibit a high degree of anther culture responsiveness. In contrast to the recalcitrant and indifferent nature of the indica subspecies, they are characterized by their resistance to callus induction (Ali et al., 2021; Mayakaduwa & Silva, 2023; Nurmansyah et al., 2021; Thi et al., 2011).

In addition to genotype, the composition of the culture medium, especially the type of basal medium and the concentration of growth regulators (such as auxins and cytokinins) as well as the carbon source, greatly affect the success of callus induction (Estaji et al., 2021; Ming et al., 2019). The utilization of an appropriate basal medium has been demonstrated to enhance the formation of callus and the proportion of callus that exhibits the potential to regenerate green plants (Ali et al., 2021; Tajedini et al., 2023). The objective of this study is to evaluate and analyze the effect of callus induction basal medium (B5 Gamborg and N6 Chu) and plant regeneration in Japonica and Indica type rice.

Method

The research was conducted at the Tissue Culture Laboratory, Department of Biotechnology, PT. BISI International, Tbk., Kediri, East Java.

An analysis of microspore development was conducted to determine the appropriate size of the panicles for use as anther culture explants. The panicle was harvested and then incubated at a pre-treatment cold temperature of 4°C for 8-9 days. The anther explants were sterilized using 70% alcohol for two minutes, 40% NaOCl for 15 minutes, and then rinsed with sterile aquades three times for three minutes each.

The sterilized panicles was then cut at the base of the grain to facilitate the anther's exit from the grain and its fall into the induction medium. The spikelets that have been collected are then tapped on the edge of a petri dish containing the induction medium. The induction medium used in this study consists of two types of basal medium (N6 Chu medium or B5 Gamborg medium), plus 50 g/L maltose, 2 g/L Gelrite, 2.5 mg/L NAA (Naphthaleneacetic Acid), 1 mg/L Kinetin, 2 mg/L 2,4-D (2,4-Dichlorophenoxyacetic Acid), 10 mg/L GA (Gum arabic) (Tajedini et al., 2023). The anthers that have been cultured are then incubated in the dark at a temperature of 25°C for 4-6 weeks until callus formation.

Callus that had formed and reached a size of 1-2 mm regenerated on MS (Murashige and Skoog) regeneration medium plus 30 g/L sucrose, 2 g/L Gelrite, 1 mg/L BAP, 1 mg/L Kinetin, and 1 mg/L NAA with pH 5.8 (Ali et al., 2021; Ahmad et al., 2016). The callus is incubated at 25°C room temperature with 16 hours of photoperiodicity. 2-3 weeks after regeneration, the plantlets formed can be green plantlets and albino plantlets. Green plantlets were then transferred to rooting media.

This study used a completely randomized design with two treatment factors, the first treatment was genotype (G) consisting of Nipponbare (Japonica-type) (G1), RCKJ 05 (Intermediate-type) (G2), RCKJ 10 (Intermediate-type) (G3), RCKJ 15 (Intermediate-type) (G4), RCKJ 25 (Intermediate-type) (Indica-type) (G5). The second factor the basal medium (M), consisting of N6 Chu medium (M1) and B5 Gamborg medium (M2).

Table 1. The Composition of N6 Chu Medium and B5 Gamborg Medium (Chu et al., 1975; Gamborg et al., 1968)

Component	Compund	N6 Medium (Chu et al., 1975)	B5 Medium (Gamborg et al., 1968)
Macronutrient	KNO_3	2830	2500
	NH_4NO_3	300	–
	$CaCl_2 \cdot 2H_2O$	440	150
	$MgSO_4 \cdot 7H_2O$	180	250
	KH_2PO_4	400	150
	K_2SO_4	1550	–
Micronutrient	H_3BO_3	100	3
	$MnSO_4 \cdot 4H_2O$	37	10
	$ZnSO_4 \cdot 7H_2O$	30	2
	$CuSO_4 \cdot 5H_2O$	1	0.025
	KI	0.83	0.75
	$Na_2MoO_4 \cdot 2H_2O$	0.25	0.25
Iron Source	$FeSO_4 \cdot 7H_2O$	27.8	27.8
	Na_2EDTA	37.3	37.3
Vitamins	Thiamine-HCl (Vitamin B_1)	1.0	10.0
	Nicotinic acid (Vitamin B_3)	–	1.0
	Pyridoxine-HCl (Vitamin B_6)	–	1.0
Other Organic Additive	–		Glycine (2.0 mg/L)

Three replicates were used for each treatment combination (genotype x medium), where one petri dish was used as a replication containing 125 anthers. Parameters observed included callus induction frequency (CIF), green plantlet regeneration (RGP), and albino plantlet regeneration (RAP). The callus induction frequency was recorded by following this formula (Ali et al., 2021):

$$CIF\ (\%) = \frac{\text{Total number of callus induced}}{\text{Total number of cultured anthers}} \text{ x } 100 \quad (1)$$

$$RGP\ (\%) = \frac{\text{Total number of green plantlets}}{\text{Total number of induced callus}} \text{ x } 100 \quad (2)$$

$$RAP\ (\%) = \frac{\text{Total number of albino plantlets}}{\text{Total number of induced callus}} \text{ x } 100 \quad (3)$$

Data were analyzed using Anova and continued with Duncan's test. Data analysis using SPSS v.25 for windows.

Result and Discussion

The Effect of Genotype and Callus Induction Media Composition on the Callus Induction Frequency

Callus induction began with selection and sterilization of explants in the laminar (Figure 1A). The selected explants were then cut and collected in sterile petridish, to facilitate the isolation of anther inside the spikelets (Figure 1B). The cut anther was then tapped on the edge of a petridish containing culture medium with two basic media treatments, namely N6 Chu medium or B5 Gamborg medium (Tabel 1). Callus formed at 4-6 weeks after anther culture (Figure 1C-D). Callus size of 1-2 mm was regenerated to regeneration media (Figure 1E). 2-3 weeks after regeneration, callus size increased and developed into green plantlets (Figure 1F).

In this study, the frequency callus induction parameter showed that the five genotypes used had different responses. The genotype RCKJ 25 (Indica-type) (G5) in B5 Gamborg callus induction basal medium showed a higher callus induction frequency response compared to N6 Chu callus induction basal medium (Figure 2A-B).

The same response occurred in three other genotypes, RCKJ 05 (Intermediate-type) (G2), RCKJ 10 (Intermediate-type) (G3), RCKJ 15 (Intermediate-type) (G4). However, the Nipponbare (Japonica-type) genotype showed the opposite response (Figure 3). For the callus induction frequency parameter, there was an interaction between genotype and callus induction medium. Nipponbare, which has a Japonica background, exhibited a higher callus induction frequency on N6 Chu medium (92.80%) compared with B5 Gamborg medium (63.47%). In contrast, RCKJ 25, which has an Indica background, showed a significant increase in callus induction frequency from only 17.87% on N6 Chu medium to 92.27% on B5 Gamborg medium.

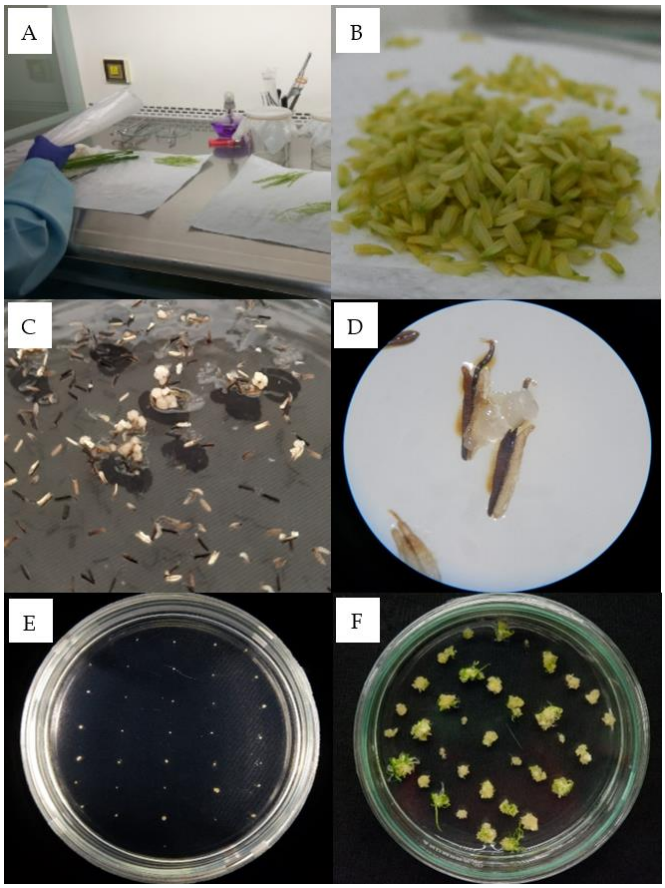

Figure 1. Double haploid induction in Rice (*Oryza sativa* L.). Explant selection (A), Spikelets cutting (B), Callus emerged from anther (C-D), Callus regeneration with size 1-2 mm (E), and Callus 1-2 weeks after regeneration (F)

This finding was particularly interesting because rice with an Indica background is generally considered recalcitrant, meaning it is typically difficult to induce callus and regenerate. However, it was able to produce significantly more callus when B5 Gamborg was used as the basal induction medium.

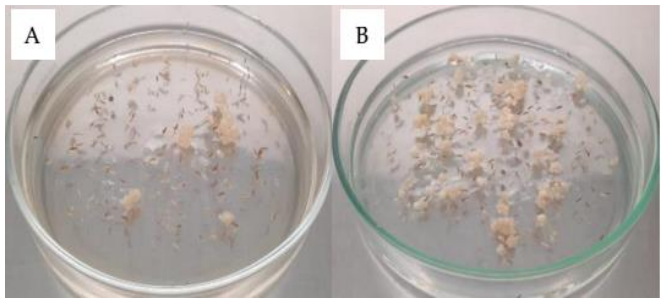

Figure 2. Callus induction frequency in RCKJ 25 (Indica-type). Callus induction in N6 Chu basal medium (A) and B5 Gamborg basal medium (B)

The other three genotypes, RCKJ 05, RCKJ 10, and RCKJ 15 with an Intermediate genetic background, which lies between Japonica and Indica, exhibited a similar pattern to RCKJ 25. Higher callus yields were obtained when B5 Gamborg was used as the basal medium for callus induction, although the percentages were not as high as those observed in RCKJ 25.

RCKJ 05 and RCKJ 10 exhibited significantly different callus induction frequencies between N6 Chu and B5 Gamborg media, with values of 6.93% and 40.00%, and 18.40% and 38.13%, respectively. However, RCKJ 15 showed no significant difference in callus induction frequency between the two media, with values of 0.00% and 4.00%, respectively.

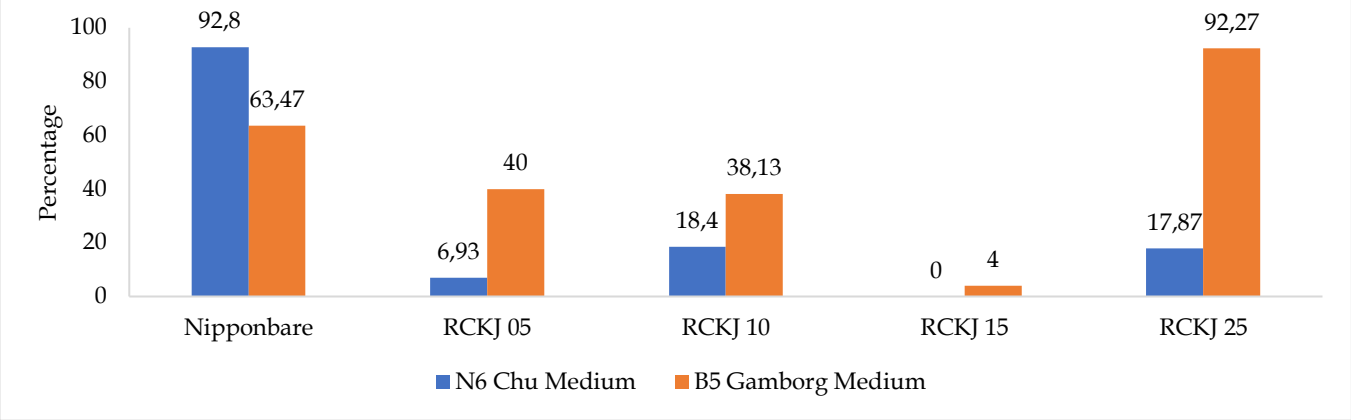

Figure 3. Effect of genotype and callus induction media composition on the callus induction frequency. Note: The same letter in each parameter indicates not significantly in the Duncan test with a real level of 5% ($p < 0.05$)

Chu et al. (1975) demonstrated N6 medium by reducing $(NH_4)_2SO_4$ and increasing KNO3 as the most suitable medium in rice anther culture. However, in this study, rice genotype RCKJ 25 showed significantly different responses. B5 medium was able to increase callus induction frequency. RCKJ 25 is an Indica-type rice. Indica-type rice has a tendency to respond differently to ammonium and nitrate. Some cultivars utilize ammonium better, while others utilize nitrate more optimally, and some show the ability to utilize both effectively (Lu et al., 2025; Abe & Futsuhara, (1985). Nitrate can inhibit cell division and cause browning. If RCKJ 25 is a genotype sensitive to high NH_4^+ (such as on N6 medium), this may inhibit cell division and cause browning.

B5 medium has a higher NO_3^-/NH_4^+ ratio, which seems to better suit the metabolic needs of Indica, favouring cell division and callus formation (Theowidavitya et al., 2019; Fatima et al., 2009). Vitamin complexes in B5, such as pyridoxine and nicotinic acid, can help in overcoming oxidative stress which is usually higher in Indica genotypes during in vitro culture (El et al., 2019; Wernicke et al., 1981).

Effect of Genotype and Callus Induction Media Composition on Callus Regeneration Potential

The callus formed has the potential to regenerate into green plantlets (Figure 4A); however, it may also develop into albino regenerants, which is a common occurrence in rice tissue culture (Figure 4B).

Calli formed from rice anther culture can develop into plantlets through the process of differentiation on regeneration media. The resulting plantlets are generally categorized into two types: green plantlets and albino plantlets (Mishra & Rao, 2016; Orłowska et al., 2020; Koetje et al., 1989).

Green plantlets originate from calli that are typically yellowish or green at the onset of differentiation. These calli develop into plantlets with healthy shoots and roots, possessing sufficient chlorophyll content to perform photosynthesis. Consequently, green plantlets are capable of surviving and growing normally after being transferred to ex vitro conditions, with high potential to develop into productive mature plants (Carsono et al., 2022; Abdullah et al., 1986).

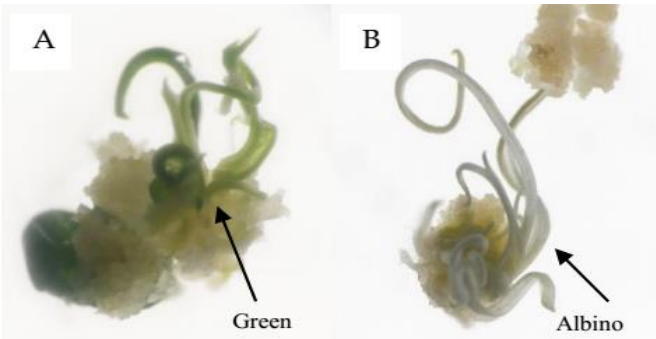

Figure 4. Callus regeneration potential. Green plantlet (A) and albino plantlet (B)

In contrast, albino plantlets arise from white or pale-yellow calli and usually undergo differentiation that favors root formation over shoot development. Due to the absence of chlorophyll, albino plantlets appear white and are incapable of effective photosynthesis. As a result, they have poor survival rates under ex vitro conditions and rarely develop into healthy adult plants (Nurhasanah et al., 2016; Callcott et al., 2018).

This study revealed an interaction effect between genotype and the basal medium used for callus induction on the regeneration capacity of green plantlets (Figure 5). The five genotypes evaluated in this study exhibited distinct responses in terms of green plantlet regeneration. Nipponbare demonstrated a higher regeneration rate on N6 Chu medium, reaching 73.49%, compared with 40.96% on B5 Gamborg medium. In contrast, genotypes RCKJ 05, RCKJ 10, and RCKJ 15 showed improved regeneration performance when cultured on B5 Gamborg medium. Specifically, green plantlet regeneration in RCKJ 05 increased from 2.38% on N6 Chu to 46.30% on B5 Gamborg; in RCKJ 10 from 10.32% to 33.35%; and in RCKJ 15 from 0.00% to 22.22%.

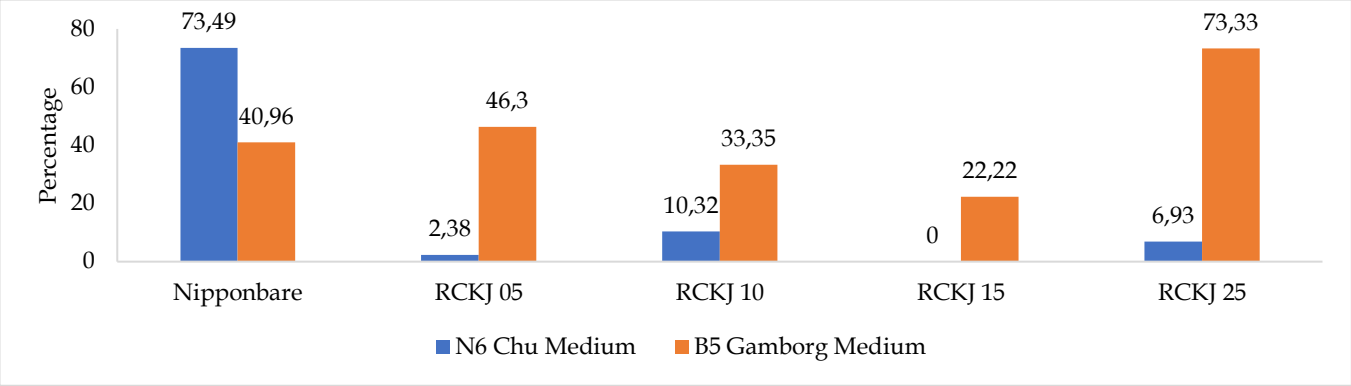

Figure 5. Effect of genotype and callus induction media composition on green plantlet regeneration (RGP). Note: The same letter in each parameter indicates not significantly different in the Duncan test with a real level of 5% ($p < 0.05$)

A highly significant difference in green plantlet regeneration was observed in the RCKJ 25 genotype. The use of B5 Gamborg as the basal induction medium resulted in a regeneration rate of 73.33%, which was substantially higher than that obtained with N6 Chu medium, which produced only 6.93% green plantlets (Figure 5).

Analysis of albino plantlet regeneration revealed no statistically significant effects of genotype, callus induction medium, or their interaction (Table 2).

Table 2. The Potency of Albino Plantlet Regeneration from Anther Cultures of Nipponbare, RCKJ 05, RCKJ 10, RCKJ 15, and RCKJ 25 Total

Genotype	Basal Medium	Total Number of Induced Callus	Number of Albino Plantlet	Albino Plantlet Regeneration (%)
Japonica	N6 Chu	116	6	5.50 a
	B5 Gamborg	79	5	6.08 a
RCKJ 05	N6 Chu	9	0	2.38 a
	B5 Gamborg	50	0	0.00 a
RCKJ 10	N6 Chu	23	0	1.04 a
	B5 Gamborg	48	0	0.00 a
RCKJ 15	N6 Chu	0	0	0.00 a
	B5 Gamborg	5	0	0.00 a
RCKJ 25	N6 Chu	22	1	3.03 a
	B5 Gamborg	115	2	1.90 a

Note: The same letter in albino plantlet indicates not significantly different based on the Duncan test with a real level of 5% ($p<0.05$).

The formation of green and albino plantlets in rice through anther culture is influenced by several key factors, including the plant genotype, the composition of the culture medium, pretreatment conditions, and the environmental conditions during culture (Kalinina & Kostylev, 2023; Zargar et al., 2022). The genotype significantly influences plantlet regeneration, with Japonica varieties generally yielding more green plantlets, while Indica varieties are more susceptible to albino plantlet formation (Niroula et al., 2005; Kojima et al., 2002). The composition of the culture medium, including the type of basal medium (such as N6 or B5), the concentration of growth regulators (such as 2,4-D and kinetin), and the carbon source (such as sucrose or

maltose) also influences chloroplast differentiation (Yan et al., 2009; Cordeiro et al., 2023).

Conclusion

Genotype and the basal medium for callus induction significantly influenced callus induction frequency and green plantlet regeneration. There is no interaction between genotype and the basal medium on albino plantlet regeneration parameter. Nipponbare, which has a Japonica background, exhibited a higher callus induction frequency on N6 Chu medium (92.80%) compared to B5 Gamborg medium (63.47%). In contrast, RCKJ 25, which has an Indica background, showed a significant increase in callus induction frequency from only 17.87% on N6 Chu medium to 92.27% on B5 Gamborg medium. A highly significant difference in green plantlet regeneration was observed in the RCKJ 25 genotype. The use of B5 Gamborg as the basal induction medium resulted in a regeneration rate of 73.33%, which was substantially higher than that obtained with N6 Chu medium, which produced only 6.93% green plantlets. These findings indicate that B5 Gamborg basal medium enhances both callus induction and green plantlet regeneration, and may be a more suitable alternative for genotypes with limited responsiveness to N6 Chu medium.

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Author Contributions
Y. F. I. contributed to research, product development, data analysis, and article writing; D. C. P., H. N. N., R. M., N. H., as a supervisor in research activities until article writing.

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Conflicts of Interest
The authors declare no conflicts of interest.

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