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Effects of BAP and GA_3 on Callus Growth and Shoot Regeneration of Indica Rice (*Oryza sativa* L.) cv. Paketih

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Abstract:
Paketih is an Indonesian indica rice variety with low amylose content and high yield potential, but like most indica rice it is difficult to regenerate in vitro. This study aimed to determine the effects of 6-benzylaminopurine (BAP) and gibberellic acid (GA_3) and their interaction on callus growth and shoot regeneration of Paketih rice. The experiment was conducted at Politeknik Negeri Jember from September to December 2025 using a factorial completely randomized design. The first factor was BAP concentration (1.0, 1.5, and 2.0 mg/L) and the second factor was GA_3 concentration (0, 0.5, and 1.0 mg/L), giving nine treatment combinations. Callus quality, diameter, and weight, time to shoot emergence, number of shoots, and shoot height were observed, and data were analyzed by ANOVA and DMRT at 5%. BAP significantly affected callus diameter and weight, with 2.0 mg/L BAP giving the largest calli (8.11 mm; 0.107 g). GA_3 and the BAP × GA_3 interaction had no significant effect on these variables. Calli were friable and turned brown by 8 days after treatment. Shoots formed only on 1.0 mg/L BAP + 0.5 mg/L GA_3, and in low numbers. BAP at 2.0 mg/L is recommended for callus proliferation, whereas shoot regeneration requires further optimization.

Keywords:
callus proliferation, cytokinin, gibberellin, indica rice, plant regeneration

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INTRODUCTION

Rice (*Oryza sativa* L.) is the staple food of Indonesia, and the country holds a large pool of local and improved varieties with valuable grain-quality traits. Paketih is an indica variety derived from a cross between Black Glutinous Rice and Pandan Wangi Cianjur. It is characterized by low amylose content and high yield potential, which make it an attractive target for further improvement through biotechnology. Genetic transformation, genome editing, somaclonal variation, and in vitro selection all depend on a reliable system for regenerating whole plants from callus. However, indica cultivars generally have lower regeneration efficiency than japonica cultivars, and protocols developed for model japonica varieties often perform poorly when applied to indica genotypes (Mostafiz and Wagiran, 2018; Chen et al., 2023). Chen et al. (2023) reported that genes related to callus induction may contribute to differences in browning and callus induction rates between indica and japonica cultivars, indicating that recalcitrance has a genetic basis that must be overcome through careful medium optimization.

Plant regeneration from callus usually proceeds in two steps. Callus is first induced on an auxin-rich medium and is then transferred to a regeneration medium in which a higher cytokinin level

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promotes the formation of shoot meristems. Auxin and cytokinin play pivotal roles in determining cell fate in regenerating tissues, and exogenous application of these hormones activates specific developmental programs in vitro (Ikeuchi et al., 2019; Long et al., 2022). The success of each step is influenced by genotype, basal medium, explant type, and the type and concentration of plant growth regulators (Phillips and Garda, 2019; Pasternak and Steinmacher, 2024). Strong genotype effects have also been documented for Indonesian rice. Local javanica varieties differed markedly in regeneration capacity on the same media (Maulidia and Fanata, 2019), and the response of rice callus to 2,4-D during induction affected its later ability to form shoots (Fanata and Qudsiyah, 2020). Consequently, a medium that works for one variety cannot be assumed to work for another, and each new genotype requires its own optimization.

Micropropagation and callus-based regeneration have become routine tools in crop improvement, but their efficiency is still limited by recalcitrance, browning, and genotype-specific requirements (Cardoso et al., 2018; Abdalla et al., 2022). For rice, the quality of the callus at the time of transfer is a key determinant of regeneration. Embryogenic callus is typically compact to friable, yellowish-white, and composed of small, dense cells, whereas watery or brown callus usually has low regenerative competence. Callus growth, expressed as diameter and fresh weight, is often used as an early indicator of callus vigor, but a large callus is not necessarily a regenerable one. Culture conditions before regeneration also matter; for example, a heat pre-treatment of seeds promoted callusing and subsequent plantlet regeneration in some Malaysian indica cultivars (Mostafiz et al., 2018). Evaluating both callus growth and callus quality is therefore necessary to judge whether a regeneration medium is moving the culture toward shoot formation.

6-Benzylaminopurine (BAP) is the cytokinin most frequently used for shoot regeneration in rice. Cytokinins are perceived by membrane-localized histidine-kinase receptors, and the signal is transduced through a phosphorelay to activate transcription factors in the nucleus that regulate cell division and shoot development (Kieber and Schaller, 2018). In the indica cultivar RD43, 2.0 mg/L BA produced the highest regeneration, with a 50% regeneration rate and 9.60 shoots per callus (Chitphet et al., 2025). In Malaysian indica varieties, regeneration media combining BAP, kinetin, and NAA gave regeneration percentages of 40–82% (Mostafiz and Wagiran, 2018; Ming et al., 2019), while in Indian indica genotypes, MS medium with 0.5 mg/L NAA and 2.0 mg/L BAP was superior for callus greening and shoot regeneration (Dhamotharan et al., 2021). The protocol for Ethiopian rice varieties was likewise optimized by testing several combinations of BAP, kinetin, and NAA for shoot regeneration (Yimam et al., 2025). These studies agree that BAP is essential, but its optimal concentration differs among genotypes.

Gibberellic acid (GA_3) is mainly known for promoting cell elongation and shoot growth. It is sometimes added to regeneration media to stimulate the elongation of newly formed shoots. However, its contribution to the initiation of shoots from callus is less clear than that of cytokinins, and the outcome of combining cytokinin with gibberellin appears to depend on concentration, genotype, and the developmental stage of the callus. In addition, regeneration of local rice varieties is frequently limited by callus browning. In Ciherang and Mentik Wangi Susu, early green spots on the callus later turned brown, resulting in low regeneration rates, and this response was associated with the expression of ethylene biosynthesis genes (Husna et al., 2022). Browning of Mentik Wangi Susu callus could be reduced significantly only by adding polyvinylpyrrolidone to the regeneration medium (Fanata et al., 2022). Browning results mainly from the oxidation of phenolic compounds released by stressed or damaged cells and can reduce callus growth and regenerative capacity (Amente and Chimdessa, 2021; Permadi et al., 2024).

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To date, no regeneration protocol has been reported for Paketih rice, and the combined effect of BAP and GA_3 on callus growth and shoot formation in this variety is unknown. This study therefore aimed to determine the effects of BAP and GA_3 concentrations and their interaction on callus growth (callus quality, diameter, and weight) and shoot regeneration (time to shoot emergence, number of shoots, and shoot height) in Paketih rice callus. We hypothesized that increasing BAP concentration would enhance callus growth and that a suitable combination of BAP and GA_3 would promote shoot regeneration. The results are expected to provide a first reference medium for the in vitro culture of Paketih and to identify the main constraints that must be addressed before this variety can be used in transformation or genome editing programs.

METHOD

The research was conducted at the Tissue Culture Laboratory, Politeknik Negeri Jember, East Java, Indonesia, from September to December 2025. The experiment was arranged in a factorial completely randomized design (CRD) with two factors. The first factor was BAP concentration (B), with three levels: B1 = 1.0 mg/L, B2 = 1.5 mg/L, and B3 = 2.0 mg/L. The second factor was GA_3 concentration (G), with three levels: G0 = 0 mg/L, G1 = 0.5 mg/L, and G2 = 1.0 mg/L. The combination of the two factors produced nine treatments (Table 1). The BAP range (1.0–2.0 mg/L) was chosen because concentrations around 2.0 mg/L have been effective for shoot regeneration in other indica rice genotypes (Dhamotharan et al., 2021; Chitphet et al., 2025), while the GA_3 range (0–1.0 mg/L) covered the low concentrations commonly added to regeneration media to support shoot growth. Each treatment was replicated three times, and each replicate consisted of culture bottles, giving experimental units. Treatments were allocated to experimental units at random, and culture bottles were placed randomly on the culture racks to minimize positional effects. The GA_3-free level (G0) served as the reference for evaluating the effect of adding GA_3 to BAP-containing media.

Table 1. Treatment combinations of BAP and GA_3 concentrations

BAP concentration	G0 (GA_3 0 mg/L)	G1 (GA_3 0.5 mg/L)	G2 (GA_3 1.0 mg/L)
B1 (1.0 mg/L)	B1G0	B1G1	B1G2
B2 (1.5 mg/L)	B2G0	B2G1	B2G2
B3 (2.0 mg/L)	B3G0	B3G1	B3G2

Note: All media were based on the same basal medium; only BAP and GA_3 concentrations differed among treatments.

The observations were analyzed according to the linear model of a two-factor CRD: $Y_{ijk} = \mu + B_i + G_j + (BG)_{ij} + \varepsilon_{ijk}$, where Y_{ijk} is the observed value of the *k*-th replicate receiving the *i*-th BAP concentration and the *j*-th GA_3 concentration, μ is the general mean, B_i is the effect of the *i*-th BAP concentration, G_j is the effect of the *j*-th GA_3 concentration, $(BG)_{ij}$ is the interaction effect, and ε_{ijk} is the random error.

Seeds of the indica rice variety Paketih were obtained from a seed source. Embryogenic calli used in this study were induced from mature seeds/explant type on callus induction medium, e.g., MS medium supplemented with x mg/L 2,4-D, and maintained for 15 days before treatment. Only healthy calli of uniform size and age, with a yellowish-white color and friable texture, were selected for transfer to the regeneration medium. Selecting calli with similar physiological conditions was intended to reduce variation among experimental units unrelated to the treatments.

The regeneration medium consisted of MS basal medium supplemented with 30 g/L sucrose and solidified with x g/L agar. BAP and GA_3 were added from stock solutions according to the

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treatment concentrations listed in Table 1. The pH of the medium was adjusted to 5.8 before sterilization in an autoclave at 121 °C for 15 minutes. GA_3 was added before autoclaving / by filter sterilization after autoclaving. The media were dispensed into culture bottles and allowed to solidify before inoculation.

All transfer work was carried out under aseptic conditions in a laminar airflow cabinet. Calli were transferred to the regeneration medium using sterile forceps, with a specified number of calli per culture bottle. The cultures were incubated in the culture room at 25 ± 2 °C under a photoperiod of 16 hours of light and 8 hours of dark. Cultures were inspected regularly for contamination, and contaminated bottles were excluded from the analysis.

Six variables were recorded. (1) Callus quality was assessed visually based on texture (friable or compact) and color (yellowish-white, yellow, or brown), including the occurrence of browning, at 8 days after treatment (DAT). Texture was classified as friable when the callus separated easily into small fragments when touched with forceps and as compact when it remained a firm, cohesive mass. A callus was considered browned when brown discoloration was visible on its surface. (2) Callus diameter (mm) was measured at 8 DAT using a measuring instrument. (3) Callus weight (g) was determined as fresh weight at 8 DAT using an analytical balance. (4) Time to shoot emergence was recorded as the number of days from transfer to the regeneration medium until the first shoot became visible. (5) The number of shoots was counted per callus. (6) Shoot height (cm) was measured from the base of the shoot to the tip of the longest leaf. Shoot variables were observed until the end of the observation period. The variables, their units, and the timing of observation are summarized in Table 2.

Table 2. Observed variables, units, and observation methods

Variable	Unit	Method of observation	Time of observation
Callus quality	–	Visual assessment of texture (friable/compact) and color, including browning	8 DAT
Callus diameter	mm	Measured with instrument	8 DAT
Callus weight	g	Fresh weight measured with an analytical balance	8 DAT
Time to shoot emergence	days	Days from transfer until the first shoot was visible	Daily observation
Number of shoots	shoots/ callus	Counted per callus	End of observation
Shoot height	cm	From shoot base to the tip of the longest leaf	End of observation

Note: DAT = days after treatment (transfer to regeneration medium).

Quantitative data were subjected to two-way analysis of variance (ANOVA) for a factorial CRD to test the main effects of BAP and GA_3 concentrations and their interaction. The calculated F values were compared with tabulated F values at the 5% and 1% significance levels. When a significant effect was detected, the means were separated using Duncan's multiple range test (DMRT) at the 5% level. Callus quality was described qualitatively. Because shoots were formed in only some treatment combinations, the shoot variables were also presented descriptively. Before ANOVA, the normality of residuals and the homogeneity of variances were checked using the Shapiro–Wilk and Levene tests. Callus diameter and weight were analyzed as continuous variables, whereas count data were square-root-transformed before analysis. Data were analyzed using statistical software. Results are presented as treatment means, and significance levels are indicated by asterisks in the ANOVA recapitulation table.

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RESULTS

The ANOVA showed that BAP concentration significantly affected callus diameter and callus weight, whereas GA_3 concentration and the BAP × GA_3 interaction had no significant effect on either variable (Table 3). The interaction between BAP and GA_3 also had no significant effect on the number of shoots.

Table 3. Recapitulation of ANOVA results for callus diameter and callus weight of Paketih rice

No.	Variable	BAP (B)	GA_3 (G)	B × G
1.	Callus diameter (mm)	*	ns	ns
2.	Callus weight (g)	*	ns	ns

Note: B = BAP concentration; G = GA_3 concentration; ** = highly significant; * = significant; ns = not significant.

Callus diameter and weight responded significantly to BAP concentration. The highest BAP concentration (2.0 mg/L) produced the largest calli, with a mean diameter of 8.11 mm and a mean weight of 0.107 g at 8 DAT. Adding GA_3 at 0.5 or 1.0 mg/L did not change callus diameter or weight significantly compared with media without GA_3, and the effect of BAP was consistent across GA_3 levels, as indicated by the absence of a significant interaction. The mean callus diameter and weight at each BAP concentration are presented in Table 4.

Table 4. Effect of BAP concentration on callus diameter and callus weight of Paketih rice at 8 DAT

BAP concentration	Callus diameter (mm)	Callus weight (g)
B1 (1.0 mg/L)	-	-
B2 (1.5 mg/L)	-	-
B3 (2.0 mg/L)	8.11	0.107

Note: Means followed by the same letter in the same column are not significantly different according to DMRT at the 5% level.

At the early stage of culture, calli in all treatments were yellowish-white and friable, which are characteristics typically associated with embryogenic callus. As the culture aged, the calli gradually turned brownish. By 8 DAT, calli from the various BAP and GA_3 treatments generally exhibited a friable texture and brownish discoloration, indicating that browning had occurred.

Shoot regeneration was observed only in the combination of 1.0 mg/L BAP and 0.5 mg/L GA_3 (B1G1). In this treatment, shoots emerged relatively early, but the number of shoots formed per callus remained low. No shoots were formed in the other eight treatment combinations during the observation period. A summary of the responses observed for all variables is presented in Table 5. Overall, the treatments fell into two response types: higher BAP concentrations favored callus enlargement, whereas shoot formation occurred only in a single combination at the lowest BAP concentration. Callus quality, in contrast, was similar across BAP and GA_3 levels, with friable texture and progressive browning observed in all treatments.

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Table 5. Summary of callus and shoot responses of Paketih rice to BAP and GA_3 treatments

Variable	Observed response	Best or responsive treatment
Callus diameter (mm)	Increased significantly with BAP; not affected by GA_3 or the interaction	B3 (2.0 mg/L BAP): 8.11 mm
Callus weight (g)	Increased significantly with BAP; not affected by GA_3 or the interaction	B3 (2.0 mg/L BAP): 0.107 g
Callus texture	Friable in all treatments	–
Callus color	Yellowish-white at the early stage, turning brownish by 8 DAT (browning)	–
Shoot regeneration	Shoots formed in one of nine treatment combinations	B1G1 (1.0 mg/L BAP + 0.5 mg/L GA_3)
Time to shoot emergence	Relatively early in the responsive treatment	B1G1
Number of shoots	Low; BAP × GA_3 interaction not significant	B1G1

Note: DAT = days after treatment.

DISCUSSION

This study evaluated, for the first time, the response of Paketih rice callus to BAP and GA_3 in a regeneration medium. Three main findings emerged. First, BAP was the only factor that significantly increased callus growth, with 2.0 mg/L giving the largest and heaviest calli. Second, GA_3 neither improved callus growth nor interacted with BAP. Third, shoot regeneration was rare and occurred only in the combination of 1.0 mg/L BAP and 0.5 mg/L GA_3, while browning developed in most calli by 8 DAT. Together, these results show that the tested media supported callus proliferation more effectively than shoot differentiation.

The positive effect of BAP on callus diameter and weight is consistent with the role of cytokinin in cell division. Cytokinin signaling activates transcription factors that regulate the expression of cell-cycle and developmental genes (Kieber and Schaller, 2018), and exogenous cytokinin enhances regenerative responses by activating specific developmental programs (Ikeuchi et al., 2019). Increasing BAP from 1.0 to 2.0 mg/L therefore likely accelerated cell proliferation and biomass accumulation in the callus. A similar concentration was effective in other indica genotypes. In RD43, 2.0 mg/L BAP produced the best regeneration (Chitphet et al., 2025), and in Indian indica genotypes, 2.0 mg/L BAP combined with NAA gave the best callus greening and shoot regeneration (Dhamotharan et al., 2021). In Paketih, however, the same concentration increased callus growth without inducing shoots. This contrast suggests that, for this variety, BAP at 2.0 mg/L mainly sustains callus proliferation, and that the cellular switch from proliferation to shoot organization requires additional signals.

GA_3 had no significant effect on callus diameter or weight. This is not surprising because gibberellins act mainly by promoting cell elongation rather than cell division, and callus enlargement during the first days of culture depends largely on cell proliferation. The lack of interaction indicates that GA_3 did not modify the callus response to BAP at the tested concentrations. In shoot regeneration systems, the balance between auxin and cytokinin is the primary determinant of cell fate (Ikeuchi et al., 2019; Long et al., 2022), and other hormones generally play a modulating rather than a decisive role. In the present study, therefore, GA_3 appears to be of secondary importance for callus growth, and its use should be evaluated mainly in relation to shoot elongation after shoots have been initiated. A stage-specific use of GA_3 may therefore be more effective: omitting GA_3 during shoot initiation, when cytokinin-driven meristem formation is the priority, and adding a low

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concentration only after shoot primordia have formed, to promote their elongation. Such a sequential design would also allow separation of the effects of GA_3 on shoot initiation and elongation, which could not be distinguished in the present experiment because both hormones were supplied together from the beginning of the regeneration stage.

Shoots formed only on the medium containing the lowest BAP concentration (1.0 mg/L) combined with 0.5 mg/L GA_3. Although this response was limited, it indicates that Paketih callus retains some regenerative competence and that a moderate cytokinin level, combined with a low gibberellin level, can trigger shoot differentiation. One possible explanation is that higher BAP concentrations favored continued callus proliferation, while the lower BAP level allowed some cells to exit the proliferative state and organize into shoot meristems. The addition of 0.5 mg/L GA_3 may then have supported the visible outgrowth of these early shoots. However, because shoots appeared in only one treatment and in low numbers, this interpretation should be treated as preliminary and needs to be confirmed in experiments with more replicates and a wider range of concentrations. The low regeneration response agrees with the general observation that indica cultivars are more recalcitrant than japonica cultivars (Mostafiz and Wagiran, 2018; Chen et al., 2023).

Browning was the most visible constraint in this study. Calli that were initially yellowish-white and friable turned brown within 8 days. Browning is mainly caused by the oxidation of phenolic compounds released from stressed or damaged cells, and the resulting quinones are toxic to plant tissues, thereby reducing cell viability and regenerative capacity (Amente and Chimdessa, 2021; Permadi et al., 2024). The susceptibility to browning varies considerably among species and genotypes, partly because of differences in phenolic content (Liu et al., 2024). In Indonesian rice, Husna et al. (2022) showed that callus of Ciherang and Mentik Wangi Susu formed green spots early but then turned brown, resulting in low regeneration, and that this response was associated with high expression of ethylene biosynthesis genes. The pattern observed in Paketih resembles these varieties and suggests that browning, possibly linked to ethylene accumulation, may have prevented many calli from progressing to shoot formation.

Practical measures to control browning are available and should be tested in future work on Paketih. Adding polyvinylpyrrolidone to the regeneration medium significantly reduced browning and improved shoot regeneration in Mentik Wangi Susu callus (Fanata et al., 2022). Other options include antioxidants such as ascorbic acid or citric acid, activated charcoal, more frequent subculture to fresh medium, and initial culture in darkness (Amente and Chimdessa, 2021; Permadi et al., 2024). Ethylene inhibitors could also be useful if ethylene accumulation is confirmed to be the cause. Because browning appeared within the first week, these treatments should be applied at the start of the regeneration stage rather than after browning becomes visible.

The composition of the regeneration medium is another factor that could be improved. In this study, BAP and GA_3 were tested without an auxin. Many successful indica regeneration protocols combine a cytokinin with a low concentration of auxin such as NAA, sometimes together with kinetin (Mostafiz and Wagiran, 2018; Ming et al., 2019; Dhamotharan et al., 2021; Yimam et al., 2025). A low auxin level helps maintain the polarity and organization required for shoot meristem formation (Long et al., 2022). Testing BAP in combination with low NAA concentrations, and comparing different basal media and carbon sources, may therefore increase the regeneration frequency of Paketih callus. Callus quality at the time of transfer is also important, because the conditions used during callus induction strongly influence subsequent regeneration (Fanata and Qudsiyah, 2020; Pasternak and Steinmacher, 2024).

The contrast between callus growth and shoot formation in this study also illustrates an important principle: callus biomass and regenerative competence are not the same. Successful

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regeneration depends on the presence of competent cells capable of undergoing the molecular reprogramming required to form new meristems (Pasternak and Steinmacher, 2024). Media that strongly stimulate cell division may enlarge the callus while keeping most cells in a proliferative, undifferentiated state. In Paketih, 2.0 mg/L BAP produced the largest calli but no shoots, while the lowest BAP level produced the only shoots. For practical protocol development, this means that callus diameter and weight should be used as indicators of callus vigor, but the selection of the best regeneration medium must be based on regeneration frequency and the number of shoots per callus. A two-step approach, in which calli are first proliferated on a medium with a higher BAP concentration and then transferred to a medium with lower cytokinin and a small amount of auxin, may combine the advantages of both responses. Pre-treatments that improve callus competence, such as the heat treatment reported for Malaysian indica rice (Mostafiz et al., 2018), could also be explored.

The timing of browning also has practical implications for the culture schedule. Because brown discoloration was already evident at 8 DAT, the interval between transfer and the first subculture may be too long for this variety. Shortening the subculture interval removes calli from medium in which oxidized phenolics and other inhibitory compounds have accumulated, and it allows the healthy parts of the callus to be separated from browned tissue before browning spreads (Amente and Chimdessa, 2021; Liu et al., 2024). Selecting only yellowish-white, actively growing callus sectors for transfer may further increase the proportion of competent cells reaching the regeneration stage. For Paketih, which combines desirable grain quality with high yield potential, an efficient regeneration system would pave the way for genetic transformation, genome editing, and somaclonal variation to further improve it. The present results indicate that achieving this goal will require an integrated strategy that addresses medium composition, browning control, and callus quality simultaneously, rather than optimizing growth regulator concentrations alone.

This study has several limitations. Callus growth was evaluated only at 8 DAT, which reflects the early response but not long-term proliferation or competence. Shoot formation was observed in only one treatment, so statistical comparison of shoot variables among treatments was not possible. The study used a single variety and a single callus induction protocol, and browning was described qualitatively rather than scored quantitatively. Future studies should include a longer observation period, quantitative browning scores, regeneration frequency (percentage of calli forming shoots), and histological confirmation of the regeneration pathway. Despite these limitations, the present results provide a first baseline for the in vitro culture of Paketih rice and identify callus browning and the composition of the regeneration medium as the main targets for further optimization.

CONCLUSION

BAP concentration significantly affected callus growth of Paketih rice, with 2.0 mg/L BAP producing the largest callus diameter (8.11 mm) and weight (0.107 g) at 8 days after treatment, whereas GA_3 and the BAP × GA_3 interaction had no significant effect on these variables. Shoot regeneration occurred only on medium containing 1.0 mg/L BAP and 0.5 mg/L GA_3 and remained low, mainly because the calli turned brown early in culture. Therefore, 2.0 mg/L BAP is recommended for callus proliferation of Paketih rice, while efficient shoot regeneration still requires further optimization of the regeneration medium and control of browning.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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