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## Plant regeneration induced from mature Embryo-derived callus of Balinese red rice (*Oryza sativa* Cv. Barak Cenana)



CrossMark

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### ABSTRACT

Recalcitrant regeneration is one of the bottlenecks for plant improvement, especially in monocotyledonous species. In this research, plantlet regeneration of a native rice cultivar *Oryza sativa* 'Barak Cenana', high-value local Balinese red rice was successfully demonstrated. Compact nodular calli were induced from excised seed-derived mature embryos of 'Barak Cenana' rice. Although 2,4-D was necessary for callus induction, a combination of 2,4-D in regeneration medium caused reduction of green spot and shoot meristemoid differentiation. Root differentiation was firstly regenerated where

meristematic shoots were thereafter developed from calli. The highest number of shoot regeneration regenerated from green spots and numbers of plantlet with complete shoot/root development were observed when transferred calli to Murashige and Skoog (MS) medium containing 5 mgL<sup>-1</sup>. Multiple shoot formation of regenerated plantlets revealed for efficient regeneration system, which was firstly reported in this present study and will potentially serve for plant improvement of this native rice, Barak Cenana.

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### INTRODUCTION

Rice is a staple food for more than half people in the world, and the demand has been predicted to increase in the future. People consume rice to gain energy as it mainly contains carbohydrates although nowadays they seek for other qualities, such as antioxidants, fragrance, texture, and etc.<sup>1</sup> Rice has become a major food commodity in several countries including Indonesia.<sup>2</sup> We can find various rice varieties and cultivars with different characteristic and quality, grown locally in certain areas, such as white rice, red rice, or black rice.

*Oryza sativa* Cv. Barak Cenana is a local Balinese red rice, cultivated in Penebel, Tabanan regency, Bali province, Indonesia.<sup>3</sup> It is considered to be a highly valued commodity since it contains vitamin B and antioxidant, a component which has the ability to prevent degenerative disease and delay aging in human, called anthocyanin.<sup>4</sup> In comparison with other types of rice varieties, vitamin B (B1 and B3), as well as anthocyanin content in Barak Cenana grain, were lower than local red rice varieties from West Java.<sup>4,5</sup> Moreover, Barak Cenana also has limitations in productivity due to its characteristic, such as long shoots, long

harvesting time and straight morphology. A tall rice is more sensitive to logging which potentially reduces harvested grain. According to Yoshida et. al, the potency of rice grain production is determined by the number of tiller.<sup>6</sup> Furthermore, it also needs six months to reach harvesting stage which makes it impossible to be cultivated for more than once a year.<sup>7</sup>

Enhancement of Barak Cenana quality and productivity potentially lead to increased production and price. The improvement of Barak Cenana can be achieved through several ways where the whole plant regeneration of this cultivar becomes very important. In this research, we would like to optimize the whole plant regeneration step of *Oryza sativa* Cv. Barak Cenana, grown in Faculty of Biotechnology, University of Surabaya greenhouse. We used mature embryo-derived calli of Barak Cenana, generated as described by Artadana et. al.<sup>8</sup> We tried to determine the preferred medium for whole plant regeneration of Barak Cenana by utilizing Murashige and Skoog (MS) medium with various plant growth regulators in different concentration.

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## RESEARCH DESIGN

This experiment was done using completely randomized design. Each bottle contains three clumps of proliferated calli which were cultured on some regeneration media with various plant growth regulators (PGR) shown in Table 1. The experiment was done using four replications for each treatment. Observation of differentiation phenomena on calli was done daily. Percentage of the green spot formed by each calli were counted after three weeks. Green spot formation is an important phenomenon to be observed since it is a first sign of the callus regeneration, which is a greenish area of callus that will differentiate into a shoot in the following days.<sup>9</sup> Furthermore, the percentage of plant regeneration was calculated as follows:

$$\text{Plant regeneration (\%)} = \frac{\text{Number of green spotted callus which grown shoot}}{\text{total number of calli each treatment}} \times 100$$

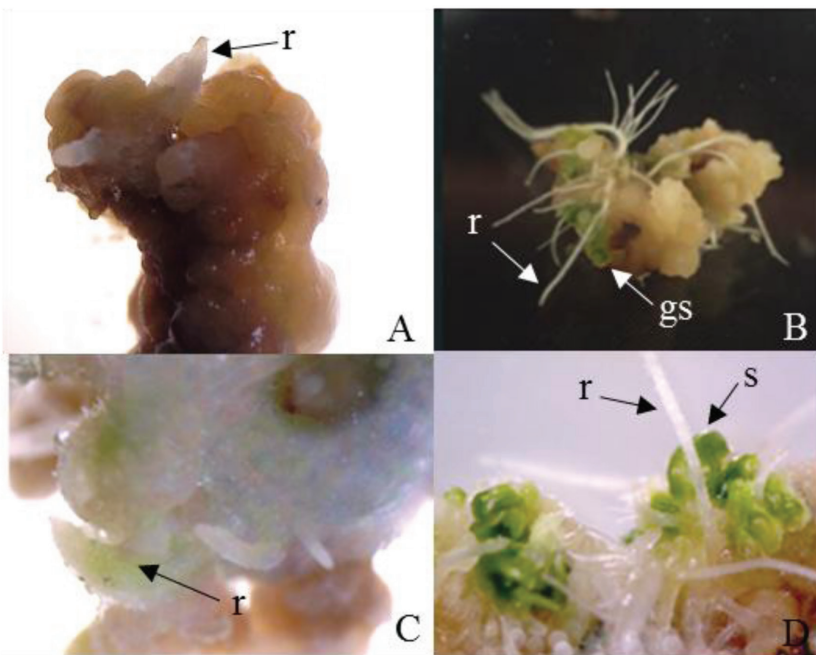
The percentage of green spotted callus and plant regeneration data was analyzed using Minitab 17 software. The mean number of green spotted callus and regenerated callus formed were compared using one-way analysis of variance (ANOVA) test and followed by Tukey test at a significant level of  $p \leq 0,05$ .

## METHODS

Mature Barak Cenana embryo-derived calli were generated using a method described by Artadana *et al.*<sup>8</sup> Sterilized-seeds were cultured in the MS medium supplemented with 1 mgL<sup>-1</sup> 2,4-dichlorophenoxyacetic acid (2,4-D). Embryo-derived callus then separated from shoot and root followed by proliferation in the MS medium supplemented with 1 mgL<sup>-1</sup> or 0.75 mgL<sup>-1</sup> 2,4-D for 20 days. After proliferation, embryo-derived calli were transferred to regeneration media contain solidified MS medium supplemented with various PGR. MS basic media was used as a control. Regenerated shoots were then separated from calli and cultured on MS basic media for further elongation. All cultures were incubated in a temperature-controlled growth room at 25°C for 16 hr of light photoperiod.

## RESULTS

Optimization of Barak Cenana calli regeneration was done by testing various concentration of different PGRs as previously used in rice or other Gramineae (e.g. sugarcane). Barak Cenana mature embryo-derived calli, generated using a method described by Artadana *et al.*, were cultured in different types of regeneration media i.e. MS basic media with additional 2 mgL<sup>-1</sup> IAA (Indole Acetic Acid), 2 mgL<sup>-1</sup> Kinetin + 0.2 mgL<sup>-1</sup> NAA (Naphthalene Acetic Acid), 5 mgL<sup>-1</sup> BAP (Benzyl Adeno Purine) + 0.8 mgL<sup>-1</sup> IAA, 3 mgL<sup>-1</sup> BAP. MS basic media without PGR was used as control.<sup>8</sup>



**Figure 1** Differentiation of Barak Cenana mature embryo-derived callus. Root formation (A), green spot and root formation (B), root differentiation from green spot (C), shoot meristemoids developed from green spot (D). Abbreviation: r: root, gs: green spot, s: shoot

**Table 1** Comparisons of callus response in different proliferation media

Proliferation media	BAP (mg L <sup>-1</sup> )	Differentiation period (days)			
		Root formation	Green spot formation	Differentiation green spot into root	Differentiation green spot into shoot
MS + 2,4-D 0.75 mg L <sup>-1</sup>	1	10 ± 0.87	29 ± 0.87	-	41 ± 1.15
	3	12 ± 0.73	22 ± 1.01	-	51 ± 1.53
	5	12 ± 0.88	20 ± 0.67	-	29 ± 0.55
MS + 2,4-D 1 mg L <sup>-1</sup>	1	11 ± 0.97	16 ± 0.67	-	-
	3	5 ± 1.00	11 ± 0.87	-	-
	5	11 ± 1,17	34 ± 0,83	-	-

We observed that root was formed within the first week in all cultures (Figure 1A). It is notably seen that root formation was longer in control and no other phenomena were observed on the following days. The green spot also appeared in certain areas in the calli from all treatments, but not in control. Additionally, green spots were regenerated to roots in MS medium supplemented with 2 mg L<sup>-1</sup> IAA or a combination of 2 mg L<sup>-1</sup> Kinetin and 0,2 mg L<sup>-1</sup> NAA. The fastest green spot formation resulted from callus on regeneration medium containing 3 mg L<sup>-1</sup> BAP.

As for the addition of BAP only in the regeneration media resulted in the fastest green spot formation, utilization of this PGR was optimized in advance. Furthermore, considering the effect of proliferation media on the whole plant regeneration, we tested calli from 1 mg L<sup>-1</sup> 2,4-D, and 0,75 mg L<sup>-1</sup> 2,4 D supplemented MS media to be cultured in regeneration media containing a various concentration of BAP. As can be seen in Table 1, we found that calli proliferated in MS + 0,75 mg L<sup>-1</sup> 2,4-D media were all successfully regenerated into the shoot. On the other hand, calli proliferated on MS + 1 mg L<sup>-1</sup> 2,4-D media only formed green spot without any further differentiation.

Based on the results, we optimized the BAP concentration on regeneration media using calli proliferated on MS + 0.75 mg L<sup>-1</sup> 2,4 D. As shown in table 2, Barak Cenana calli were cultured in MS medium containing a wider concentration of cytokinin BAP which was 1, 3, 5, and 6 mg L<sup>-1</sup>. It is notably seen that green spot in all BAP

supplemented media were successfully differentiated into the shoot. We found that the highest concentration of BAP, 6 mg L<sup>-1</sup>, induced the fastest green spot formation (on day 16) and differentiation (on day 20) followed by other lower concentrations (Table 2). Moreover, we also tested the effect of BAP and TDZ (Tidiazuron) combination on regeneration media. We observed that the green spot formation on calli cultured in this medium was faster than MS + BAP 6 mg L<sup>-1</sup>. However, the combination of these PGRs was unsuccessful to generate shoot from the green spot.

We calculated the percentage of green spotted calli and plant regeneration as well as the average number of shoots to determine the most potent concentration of BAP in regeneration media (Table 3). We found that the difference in BAP concentration did not significantly affect shoot regeneration. The highest average number of shoots formed was 8 per callus clump, achieved by culturing on MS + 5 mg L<sup>-1</sup> and 6 mg L<sup>-1</sup> BAP. As a comparison, calli cultured on MS media only showed the increase of callus number instead of regeneration phenomena (data not shown). Nevertheless, the shoot regeneration from calli grown in MS + 5 mg L<sup>-1</sup> media were more consistent than calli grown in MS + 6 mg L<sup>-1</sup> media as shoot regeneration was achieved in every replication in MS + 5 mg L<sup>-1</sup> BAP whether we only observed one in each replication of MS + 6 mg L<sup>-1</sup> media.

In general, we observed that the plant regeneration of Barak Cenana mature embryo-derived callus, initiated by BAP induction, was conducted

**Table 2** Callus response in regeneration media containing cytokinin only

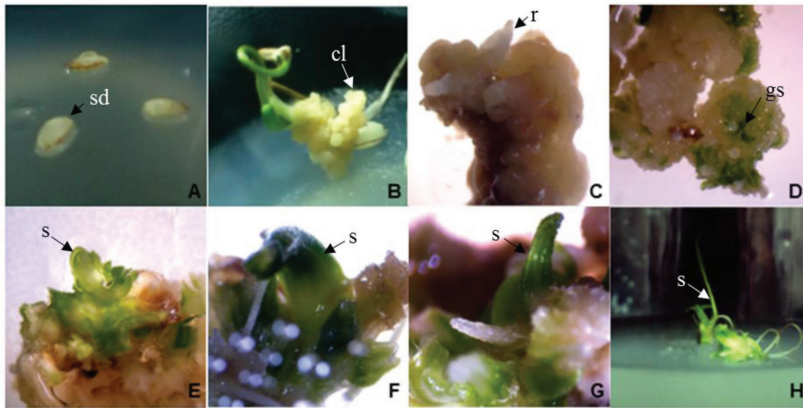
Cytokinin concentration (mg L <sup>-1</sup> )	Differentiation period (days)			
	Root formation	Green spot formation	Differentiation green spot into root	Differentiation green spot into shoot
MS + 1 mg L <sup>-1</sup> BAP	12 ± 0.73	22 ± 1.01	-	51 ± 1.53
MS + 3 mg/L BAP	10 ± 0.87	29 ± 0.87	-	41 ± 1.15
MS + 5 mg/L BAP	12 ± 0.88	20 ± 0.67	-	29 ± 0.55
MS + 6 mg/L BAP	8 ± 0.88	16 ± 1.41	-	20 ± 0.55
MS + 5 mg/L BAP + 0.2 mg/L TDZ	8 ± 0.50	15 ± 0.50	22 ± 0.58	-

**Table 3** Percentage of green spotted callus, plant regeneration, and the number shoot formed by calli on some regeneration media

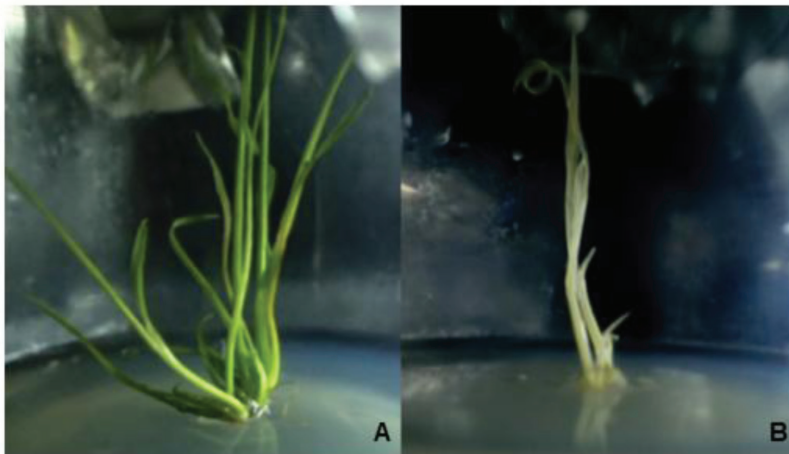
Regeneration media	Green spotted callus (%)	Plant regeneration (%)	Highest number of shoot	Average number of shoot*
MS + 1 mg L <sup>-1</sup> BAP	60.0 ± 10 <sup>a</sup>	12.3 ± 11	8	5
MS + 3 mg L <sup>-1</sup> BAP	50.0 ± 10 <sup>a</sup>	20.7 ± 4	3	2
MS + 5 mg L <sup>-1</sup> BAP	56.7 ± 12 <sup>a</sup>	29.7 ± 10	23	8
MS + 6 mg L <sup>-1</sup> BAP	36.7 ± 15 <sup>ab</sup>	20.0 ± 35 <sup>ns</sup>	14	8

\* in one callus clump. Numbers followed by different letter in the same column differed significantly according to Tukey test with 5% α.





**Figure 2** Whole plant regeneration of mature embryo-derived Barak Cenana calli. Barak Cenana seeds (A). Barak Cenana calli (B) Root formation in calli (C) Green spot formation in rooted calli (D) green spot derived shoots (E-G) *in vitro* whole plant (H)



**Figure 3** Comparison between Barak Cenana normal (A) and albino (B) plant

through indirect organogenesis (Figure 2). The regeneration was initiated by root formation followed by green spots formation. Those green spots were grown wider and darker which remarked the shoot differentiation. The shoots continued to elongate and generate an *in vitro* whole plant with a tiller. We also have been successfully acclimatized the *in vitro* whole plant (data not shown).

Moreover, we found other phenomena such as some shoots generated from MS + 5 mg/L media were more vigorous than others and an albino shoot. The albino shoot resulted from a callus grown on MS + 1 mg/L BAP (Figure 3). This structure was similar to root structure according to the color. However, the straight costa pattern showed that it was albino shoot. The albino shoot was cultured on MS basic media for further elongation. This character was persistent even after three times subculture indicating a somaclonal variation.

## DISCUSSION

Cellular differentiation and morphogenesis in plant tissue culture are controlled by the interaction of auxin and cytokinin at certain ratio.<sup>10</sup> The balance of these two plant growth regulators is necessary for differentiation in plant tissue culture. The combination of auxin and cytokinin usually used to regenerate some cultivars of embryogenic callus rice.<sup>11</sup> Sahnó et al. showed that combination of 0,2 mg/L NAA and 2 mg/L Kinetin could regenerate callus from indica rice cultivars such as IR64, PB1, CSR10, and Swarna.<sup>12</sup> Rattana et al. successfully regenerated callus from rice cultivar Chai Nat 1 using a combination of 0,5 mg/L NAA and 5 mg/L BAP.<sup>13</sup> In 2012, Rattana et al. were successful to regenerate rice cultivar Khao Dawk Mali 105 and Patum Thani 1 calli using 3 mg/L and 5 mg/L BAP, respectively.<sup>13</sup> Maftucah also reported that a successful regeneration of Cisadane rice was achieved using 0,7 mg/L IAA + 0,5 mg/L BAP.<sup>14</sup> In another Gramineae, utilization of 2 mg/L IAA was successfully regenerated sugar cane callus.<sup>15</sup> In this research, we tried the addition of one or various combination of PGRs, auxin (IAA, NAA) and cytokinin (BAP, Kinetin, TDZ) to regenerate Barak Cenana mature embryo-derived callus. We found that Barak Cenana calli were successfully regenerated using MS media supplemented only with BAP while the other treatments were unable to exhibit regeneration phenomena. This indicates that the composition regeneration media is species specific.<sup>10</sup>

During observation, we saw that calli in MS basic media without any PGR supplementation generated roots. This can be caused by the remaining 2, 4-D which has not been degraded or converted and stored inside the calli.<sup>16</sup> Considering this factor, we did a further experiment on the effect of 2,4-D in proliferation media to the regeneration process. The result showed that calli proliferated in media with a lower concentration of 2,4-D in proliferation media (0.75 mg/L) were successfully regenerated into shoot while calli in 1 mg/L 2,4-D were not able to form shoots at all. This phenomenon was similar to the results obtained by Al-Khayri and Al-Bahrany as well as Malik et al. which showed that the decrease in 2,4-D concentration in induction and proliferation media could enhance the success of rice callus regeneration process as well as increasing the possibility of callus regeneration into the shoot, respectively.<sup>17,18</sup>

Optimization of BAP concentration in Barak Cenana regeneration media was conducted with MS basic media supplemented with 1, 3, 5, and 6 mg/L BAP. All of these concentrations were able to regenerate red rice cultivar Barak Cenana calli

into the shoot. Based on the percentage of green spotted callus which grown shoot, MS + 5 mg/L increased the success of Barak Cenana callus regeneration. On the other hand, BAP concentration at 6 mg/L decreased the success rates of Barak Cenana callus regeneration. This trend is similar to Malaysian rice, as reported by Htwe et al., where the regeneration capability is also decreased after the increase of BAP above 10  $\mu$ M.<sup>19</sup> In comparison to Patum Thani 1, the addition of 5 mg/L BAP in Barak Cenana callus regeneration media resulted in a higher average number of shoots than Patum Thani 1 although the percentage of plant regeneration is 2.3% lower than Patum Thani 1.<sup>13</sup> We also tried to add 0.2 mg/L TDZ, a potent cytokinin, in combination with 5 mg/L BAP where this media failed to regenerate callus into the shoot. According to Bhojwani & Razdan, too high concentration of PGR in the plant tissue culture media will lower the regeneration ability.<sup>20</sup> Therefore, we can conclude that the optimum concentration obtained for Barak Cenana mature embryo-derived callus regeneration was on 5 mg/mL BAP.

Plant regeneration is initiated once the green spot, areas that turned into greenish and will form adventitious shoot later (Purnamaningsih, 2003), is formed.<sup>9</sup> Green spot is commonly regenerated into shoot although some of it can differentiate into root depending on the green spot anatomical structure that resembled root apices.<sup>21</sup> In general, whole plant regeneration of Barak Cenana mature embryo-derived calli is similar to rice cultivar Sita, Rupali, Masuri, IR64, PB1, CSR10, and Swarna.<sup>12,22</sup> We also noticed that shoot formation time of Barak Cenana callus is quite similar with Sambha mahsuri callus which is successfully regenerated from a shoot using MS + 5 mg/L BAP media on the 27<sup>th</sup> day.<sup>23</sup> Furthermore, the shoots regenerated from Barak Cenana mature embryo-derived calli are successfully developed into *in vitro* whole plant in MS basic media and typically grown during acclimatization.

In addition, we found a phenomenon of albino shoots where this character is persistent after three times subculture. Puan and Siddiq showed that this phenomenon also occurred in Pusa Basmati, IR-64, and Jaya rice calli which were cultured on MS basic media supplemented with 0,25 mg/L BAP.<sup>24</sup> As reported by George, the albino characteristic is caused by the reduction of chlorophyll formation ability due to the presence of 2, 4-D auxin in callus cultured on MS basic medium supplemented with a low concentration of BAP.<sup>10</sup> Thus, we conclude that this albino shoot is a permanent soma clonal variation due to its consistency after subculturing in MS basic media without 2,4-D.

## CONCLUSION

In summary, Barak Cenana mature embryo-derived calli were previously proliferated in MS + 0.75 mg/L 2,4-D followed by culturing in MS + 5 mg/L BAP as the optimum regeneration media to achieve whole plant regeneration. This regeneration media also potential to induce soma clonal variation on Barak Cenana calli as we observed other characteristics such as vigor shoots and albino shoots.

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