

# Enhancing the tolerance of local Badui rice (*Oryza sativa* L.) to salinity stress through a combination of induced variation and in vitro selection

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**Abstract.** Rice (*Oryza sativa* L.) is one of the most important food crops, but its productivity is threatened by the widespread conversion of agricultural land. One way to address this issue is to use suboptimal land, such as saline land. However, high soil salinity can significantly reduce productivity. Efforts to address this challenge involve developing salt-tolerant rice varieties using variation induction and in vitro selection methods. This study aims to determine the optimal combination of plant growth regulators (PGRs) for the induction and growth of local Badui rice callus and to obtain a callus that exhibits resistance to salinity stress. Local Badui rice callus was induced on Chu N6 medium with various combinations of PGRs and then selected on salinity stress selection medium with NaCl concentrations of 2500–7000 ppm. The results showed that the optimal induction medium for callus formation was achieved with the addition of 2,4-D at 2.5 ppm, yielding the highest callus formation rate of 84% and the combination of NAA at 1.5 ppm + BAP at 0.25 ppm resulted in the fastest callus formation at 7.01 days post-planting. Meanwhile, the medium with the addition of 2,4-D at 1 ppm was the optimal callus growth medium, yielding the highest average callus mass and diameter, namely 204 mg and 12.5 mm, respectively. Local Badui rice callus was proven capable of surviving at NaCl concentrations up to 7,500 ppm with a survival rate of 100%.

## 1 Introduction

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Rice (*Oryza sativa* L.) is the staple food for over 50% of the world’s population and more than 90% of Indonesians, making it crucial for food security. However, rice production in Indonesia has declined, partly due to land conversion, forcing cultivation on suboptimal areas such as saline soils [1]. Salinity, characterized by high Na<sup>+</sup> and Cl<sup>-</sup> levels, disrupts water balance, nutrient uptake, and growth, and rice, as a glycophyte, is particularly sensitive to this stress [2]. To address this, the development of salinity-tolerant rice varieties is essential. Conventional breeding and genetic transformation are widely used but constrained by land requirements, long time frames, and potential biosafety issues. By contrast, induced variation combined with in vitro selection offers a more efficient, safer, and land-saving alternative [3]. In vitro culture enables controlled callus induction and the generation of somaclonal variation, providing a basis for selecting stress-tolerant variants [4].

In rice, N6 basal medium supplemented with auxins such as 2,4-D or NAA, alone or combined with cytokinins like BAP, is commonly used for callus induction. Among these, 2,4-D is particularly effective in stimulating callus formation and somaclonal variation due to its stability and high affinity for auxin transporters [5]. The callus induction stage is critical for generating genetic variation, which can then be directed toward salinity tolerance through in vitro selection using NaCl as a selective agent [6].

Local Badui rice is a genetically diverse upland landrace with desirable agronomic traits and high potential as a genetic resource, yet studies on optimal plant growth regulators for callus induction and the development of salinity-tolerant variants are still limited [7]. This study therefore aimed to determine the optimal callus induction medium for local Badui rice and to obtain calli tolerant to salinity stress using NaCl as a selection agent, thereby contributing to the development of salinity-tolerant varieties and supporting food security and SDG Goal 2, Zero Hunger.

## 2 Research Method

### 2.1 Preparation of Culture Media

Two types of N6-based media were used, namely callus induction medium and selection medium. The callus induction medium was prepared by supplementing N6 basal medium (3.96 g L<sup>-1</sup>) with sucrose (30 g L<sup>-1</sup>), agar (8 g L<sup>-1</sup>), and plant growth regulators at various concentrations as presented in **Table 1**.

**Table 1.** Combinations of N6 basal medium and concentrations of plant growth regulators

| Treatment | Basal Medium | Plant Growth Regulators    |
|-----------|--------------|----------------------------|
| P.1       | N6           | -                          |
| P.2       | N6           | 2,4-D 1 ppm                |
| P.3       | N6           | 2,4-D 2,5 ppm              |
| P.4       | N6           | 2,4-D 5 ppm                |
| P.5       | N6           | NAA 1,5 ppm                |
| P.6       | N6           | NAA 1,5 ppm + BAP 0,25 ppm |

The selection medium used the most optimal formulation from the callus induction stage and was further supplemented with NaCl at 0, 2500, 5000, and 7500 ppm. Media preparation involved dissolving N6 and sucrose in distilled water, adding the appropriate PGRs, adjusting the pH to 5.8 ± 0.2, adding agar, and dispensing the medium into culture bottles.

### 2.2 Sterilization of Media, Equipment, and Materials

Culture media, glassware, solutions, and supporting materials were wrapped and sterilized by autoclaving at 121°C and 15 psi for 20 minutes. Sterilized media were incubated for 2 days to confirm sterility before use.

## 2.3 Sterilization of Explants

Dehulled rice seeds were prewashed in detergent solution and rinsed thoroughly. In a laminar airflow cabinet, seeds were sterilized with 70% ethanol, followed by 2% NaOCl with Tween 20, then rinsed three times with sterile distilled water prior to inoculation.

## 2.4 Callus Induction

Sterile seeds were placed on callus induction media (5 bottles × 5 explants per treatment) and incubated in the dark. Initial callus formation was observed for 28 days, followed by a 28-day subculture on the same medium. Callus induction percentage, color, texture, fresh weight, and diameter were recorded, and the optimal medium was selected based on high induction, vigorous growth, and white–yellow callus morphology.

## 2.5 Selection for Salinity Stress in Rice Calli

Calli with white to yellow coloration and ~100 mg fresh weight were transferred to selection media containing 0, 2500, 5000, or 7500 ppm NaCl (3 bottles × 3 calli per treatment). Cultures were incubated in the dark for 28 days, then evaluated for survival, color, texture, fresh weight, diameter, and growth rate.

## 3 Result and Discussions

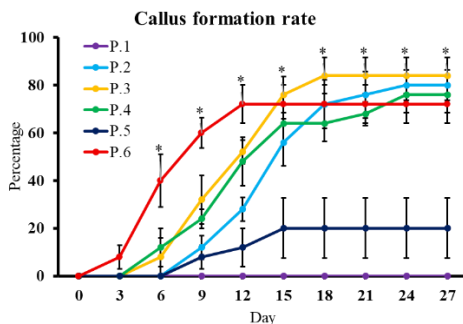
### 3.1 Effect of Plant Growth Regulator Combinations on the Callus Induction Percentage and Callus Formation Rate of Local Badui Rice

**Table 2.** Effect of Plant Growth Regulator (PGR) Combinations on Callus Induction, Growth, and Morphology of Local Badui Rice.

| Treatment | PGR        |           |           | Percentage callus induction (%) | Callus mass (mg)       | Callus diameter (mm)    | Callus formation time (days) | Callus characteristics                   |
|-----------|------------|-----------|-----------|---------------------------------|------------------------|-------------------------|------------------------------|--|
|           | 2,4D (ppm) | NAA (ppm) | BAP (ppm) |                                 |                        |                         |                              |  |
| P.1       | -          | -         | -         | 0,00 ± 0,00 <sup>b</sup>        | -                      | -                       | -                            | -  |
| P.2       | 1          | -         | -         | 80,00 ± 6,32 <sup>a</sup>       | 204 ± 18 <sup>a</sup>  | 12,5 ± 8 <sup>ns</sup>  | 204 ± 18 <sup>a</sup>        | Friable and pale yellowish-white         |
| P.3       | 2,5        | -         | -         | 84,00 ± 7,48 <sup>a</sup>       | 162 ± 15 <sup>ab</sup> | 10,5 ± 5 <sup>ns</sup>  | 162 ± 15 <sup>ab</sup>       | Friable and pale yellowish-white         |
| P.4       | 5          | -         | -         | 76,00 ± 7,48 <sup>a</sup>       | 135 ± 28 <sup>b</sup>  | 9,8 ± 18 <sup>ns</sup>  | 135 ± 28 <sup>b</sup>        | Friable and pale yellowish-white         |
| P.5       | -          | 1,5       | -         | 20,00 ± 8,94 <sup>b</sup>       | 40 ± 23 <sup>c</sup>   | 10,0 ± 33 <sup>ns</sup> | 40 ± 23 <sup>c</sup>         | Compact, black, and hairy (root forming) |
| P.6       | -          | 1,5       | 0,25      | 72,00 ± 8,00 <sup>a</sup>       | 115 ± 4 <sup>b</sup>   | 7,3 ± 3 <sup>ns</sup>   | 115 ± 4 <sup>b</sup>         | Compact and black                        |

Explant rice seeds were cultured on N6 medium with various plant growth regulator (PGR) combinations. Callus induction percentage and callus formation rate are summarized in **Table 2** and **Fig. 1**. The highest callus induction was obtained in P.3 (84%), followed by P.2 (80%), P.4 (76%), and P.6 (72%), which were not significantly different ( $p > 0.05$ ). P.1 showed no callus formation (0%), while P.5 had a low induction (20%) and, together with P.1, differed significantly from the other treatments ( $p < 0.05$ ). Callus formation rate varied among treatments, with P.6 showing the fastest initiation (7.01 days) and P.2 the slowest (13.70 days). P.3, P.4, and P.5 had intermediate initiation times (around 10–12 days), with P.6 significantly different from all other treatments ( $p < 0.05$ ). Over 27 days of observation (**Fig. 1**), P.6 exhibited the earliest and rapid response, reaching its maximum (72%) by day 12, whereas P.3 ultimately achieved the highest final induction. P.2 and P.5 initiated callus

later (day 9), with P.5 plateauing at 20%. No callus was observed in P.1 throughout the experiment.



**Fig. 1.** Graph of callus formation rate based on different PGRs over 27 days.

Data represent the mean of 5 replicates. The asterisk (\*) on the graph indicates a significant difference between treatments at the same time point according to ANOVA ( $p < 0.05$ ).

### 3.2 Effect of Plant Growth Regulator Combinations on Callus Growth of Local Badui Rice

Calli formed during the induction stage were transferred to fresh media with the same plant growth regulator combinations and incubated for 28 days, after which fresh weight and diameter were measured (**Table 2**). The highest mean callus mass was obtained in treatment P.2 (204 mg), while the lowest was in P.5 (40 mg). Treatment P.2 differed significantly from P.4, P.5, and P.6, but not from P.3. For diameter, P.2 also showed the largest mean value (12.5 mm), and P.6 the smallest (7.3 mm), although no significant differences among treatments were detected. Calli in 2,4-D treatments (P.2, P.3, P.4) were generally friable and white to yellowish, whereas NAA (P.5) induced browning and root organogenesis, and the NAA + BAP combination (P.6) produced compact, browned calli.

### 3.3 Effect of Salinity Stress on Callus Morphology and Callus Survival Percentage

Rice calli were subcultured onto media supplemented with NaCl and incubated for 28 days to evaluate their response to salinity stress, as shown in **Table 3**. All treatments showed 100% callus survival, but browning increased with higher NaCl concentrations. No browning occurred in S.1, while browning reached 1.70% in S.2, 7.60% in S.3, and 24.63% in S.4. Treatments S.1, S.2, and S.3 did not differ significantly, but all were significantly different from S.4 ( $p < 0.05$ ), indicating that higher NaCl levels promote callus browning.

### 3.4 Effect of Salinity Stress on Callus Mass and Diameter Growth

Rice calli subcultured on salinity stress media for 28 days showed a decline in fresh mass increment and growth rate with increasing NaCl concentration as presented in **Table 3**. Treatment S.1 (0 ppm NaCl) had the highest mass increment (220.33 mg) and growth rate (260.10%), while S.4 (highest NaCl level) showed the lowest values (127.33 mg; 93.15%). Treatments S.2, S.3, and S.4 did not differ significantly from each other but were all significantly lower than S.1 ( $p < 0.05$ ). A similar trend was observed for callus diameter. The greatest diameter increase occurred in S.1 (6.33 mm), whereas S.4 showed the smallest increment (1.74 mm). Statistical analysis confirmed that S.1 differed significantly from the other treatments.

**Table 3.** Effect of salinity stress on callus of local Badui Rice

| Treatment | NaCl concentration (ppm) | Percentage of surviving callus | Percentage of browning callus | Rate of callus growth       | Callus mass increment (mg)  | Callus diameter increment (mm) |
|-----------|--------------------------|--------------------------------|-------------------------------|-----------------------------|-----------------------------|--------------------------------|
| S.1       | 0                        | 100                            | 0,00 ± 0,00 <sup>a</sup>      | 260,10 ± 56,92 <sup>a</sup> | 220,33 ± 23,97 <sup>a</sup> | 6,33 ± 0,22 <sup>a</sup>       |
| S.2       | 2500                     | 100                            | 1,70 ± 0,85 <sup>a</sup>      | 142,94 ± 8,28 <sup>b</sup>  | 151,00 ± 4,73 <sup>b</sup>  | 3,71 ± 0,69 <sup>b</sup>       |
| S.3       | 5000                     | 100                            | 7,60 ± 3,50 <sup>a</sup>      | 126,74 ± 26,62 <sup>b</sup> | 128,33 ± 20,22 <sup>b</sup> | 2,30 ± 0,62 <sup>bc</sup>      |
| S.4       | 7500                     | 100                            | 24,63 ± 3,30 <sup>b</sup>     | 93,15 ± 28,90 <sup>b</sup>  | 127,33 ± 19,85 <sup>b</sup> | 1,74 ± 0,64 <sup>c</sup>       |

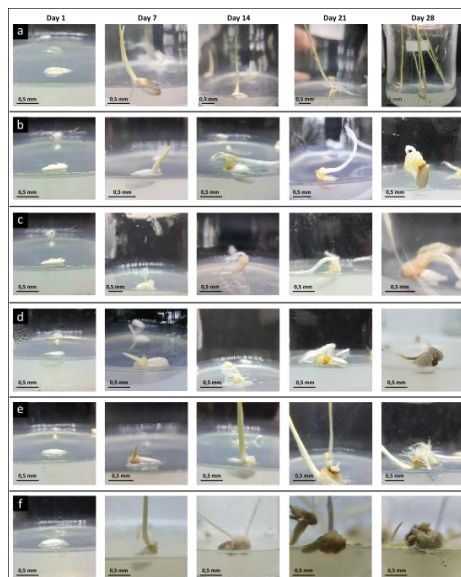
### 3.5 Forming Rate and Growth of Local Badui Rice Callus on In Vitro Culture Media with Different Plant Growth Regulator Combinations

Callogenesis is the trans-differentiation of plant explants into unorganized cell masses (calli) regulated mainly by auxins and cytokinins [8]. In this study, Badui rice seeds formed calli under all PGR-supplemented treatments, with callus induction ranging from 20% with NAA 1.5 ppm to 84% with 2,4-D 2.5 ppm, while no callus developed on N6 medium without PGRs. These results indicate that both PGR type and concentration strongly affect callus induction [9], with 2,4-D more effective for callus formation in cereals and NAA favoring root organogenesis due to differences in their signaling pathways [10]. Notably, 2,4-D primarily stimulates callus formation in cereals, whereas NAA favors root induction due to differences in signaling pathways

Callus formation rates varied among treatments. The fastest induction occurred with NAA 1.5 ppm + BAP 0.25 ppm (7.01 days), whereas 2,4-D 1 ppm showed the slowest induction (13.70 days). The treatment with the highest callus percentage (2,4-D 2.5 ppm) had an average formation time of 10.47 days. Callus initiation was observed approximately 7 days after sowing, beginning at the shoot base, as shown in **Fig. 2**, indicating the early stage of callus development, consistent with previous findings that callus formation starts from the scutellum and mesocotyl via localized cell division [11].

Initially, calli appeared translucent white and developed into pale yellowish-white by day 14. Callus texture differed between treatments: 2,4-D treatments produced friable calli (soft, easily separated, high water content), whereas NAA + BAP treatments yielded compact calli (hard, difficult to separate, low water content). NAA treatments also produced friable calli, with some undergoing organogenesis into rooty calli around day 21, with root number increasing by day 28.

Callus growth was optimal in Chu N6 medium with 2,4-D 1 ppm, which produced the highest average callus mass (204 mg) and diameter (12.5 mm), while 2,4-D 5 ppm reduced growth, likely due to inhibition of cell division [12]. NAA alone caused browning and root formation, whereas NAA + BAP induced severe browning and black calli, probably due to excessive phenolic accumulation driven by BAP activation of polyphenol oxidase and phenolic biosynthesis [13]. Overall, 2,4-D 1 ppm is optimal for callus proliferation in local Badui rice, while NAA-based treatments are more suitable for root organogenesis.



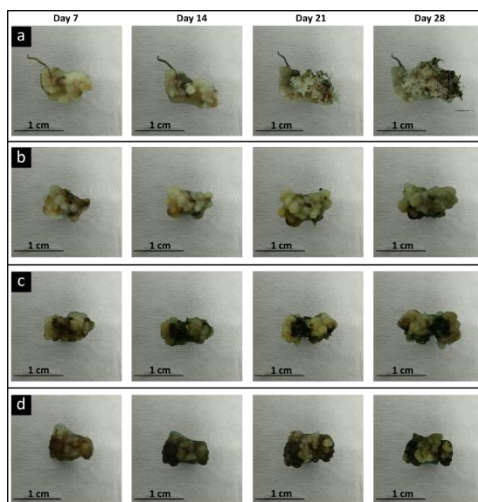
**Fig. 2** Growth of local rice seed explants on in vitro culture media  
(a. N6; b. N6 + 2,4-D 1 ppm; c. N6 + 2,4-D 2.5 ppm; d. N6 + 2,4-D 5 ppm; e. N6 + NAA 1.5 ppm; f. N6 + NAA 1.5 ppm + BAP 0.25 ppm)

### 3.6 Viability and Growth of Local Badui Rice Callus under Salinity Stress

Salinity stress arises from high concentrations of dissolved salts, especially  $\text{Na}^+$  and  $\text{Cl}^-$ , and in tissue culture  $\text{NaCl}$  is commonly used to raise medium salinity. In this study, local Badui rice calli showed 100% survival at all  $\text{NaCl}$  levels tested (0–7500 ppm), indicating tolerance and absence of lethality at 7500 ppm. In contrast, Luam Pua rice calli previously showed reduced survival from 76.66% at 50 mM  $\text{NaCl}$  to 30% at 200 mM  $\text{NaCl}$ . At higher concentrations, the viability of Luam Pua calli further declined to 33.33% at 150 mM and 30% at 200 mM  $\text{NaCl}$  [14].

The ability of calli to tolerate salinity stress is attributed to defense mechanisms, one of which is the accumulation of osmolytes such as sucrose, proline, glycine betaine, and others [16]. In the previous study by, proline content in Pokkali rice calli was 14.88  $\mu\text{mol/g}$  under control conditions, which increased progressively with  $\text{NaCl}$  concentration, reaching 183.77  $\mu\text{mol/g}$  at 2.0%  $\text{NaCl}$  [16]. Osmolytes play a crucial role in salinity stress by balancing osmotic pressure between the extracellular environment and the cell.

Local Badui rice calli survived salinity stress up to 7500 ppm  $\text{NaCl}$  but showed morphological changes, particularly in color. Calli in the control medium were white to yellowish, whereas calli on selective media became increasingly brown over time, especially at 5000 and 7500 ppm  $\text{NaCl}$ , as shown in **Fig. 3**. Similar browning responses have been reported in maize calli exposed to  $\text{NaCl}$ . This browning results from the accumulation and oxidation of phenolic compounds [17], which, although part of the plant's defense under stress, can cause tissue necrosis and degradation when excessively accumulated [13].



**Fig. 3.** Callus morphology on selection media with various NaCl concentrations (a. NaCl 0 ppm; b. NaCl 2500 ppm; c. NaCl 5000 ppm; d. NaCl 7500 ppm)

Salinity in the selection medium markedly reduced the growth of local Badui rice calli. The control treatment showed the highest mean mass increase (220.33 mg), diameter increment (6.33 mm), and growth rate, all of which declined with increasing NaCl concentration, reaching 127.33 mg and 93.15% at 7500 ppm NaCl. NaCl lowers the osmotic potential of the medium, causing osmotic stress that disrupts nutrient uptake and turgor pressure, thereby inhibiting callus growth. In addition, excessive  $\text{Na}^+$  and  $\text{Cl}^-$  accumulation leads to ion toxicity [18]. While high  $\text{Na}^+$  interferes with  $\text{K}^+$  uptake, which is essential for ionic balance and membrane stability, potentially causing water loss in calli [17].

## 4 Conclusion

The optimal PGR for callus induction in local Badui rice was 2,4-D 2.5 ppm, which produced the highest callus formation (84% at 10.47 days after planting). In terms of speed, NAA 1.5 ppm + BAP 0.25 ppm was most effective, inducing 72% callus formation within 7.01 days. For callus growth, 2,4-D 1 ppm was optimal, yielding the highest callus mass (204 mg) and diameter (12.5 mm). Local Badui rice calli also survived on media containing up to 7500 ppm NaCl, with 100% survival despite reduced mass, growth rate, and diameter, indicating strong salinity tolerance.

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