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Effect of Plant Growth Regulators on Callus Induction of Black Rice (*Oryza sativa* L.)

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Authors' contributions

This work was carried out in collaboration among all authors. Authors BR and RSD designed the study, authors BR and UM supervise the whole progress of the research work. Authors GSS and RSD performed the statistical analysis, author RSD wrote the protocol and wrote the first draft of the manuscript. Author RSD managed the literature search. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Aims: Black rice of Manipur is known for its aroma and nutritional quality. As per the reports on tissue culture of rice, callus induction response of traditional cultivars was much lower than the modern high yielding varieties. Considering the importance of the black rice, and effort was taken to standardized callus induction protocol of black rice of Manipur.
Study Design: Complete Randomized Design.
Place and Duration of Study: Department of Seed Science and Technology, Faculty of Agriculture, Uttar Banga Krishi Viswavidyalaya, Pundibari, Cooch Behar 736165, West Bengal, India, between June 2020 and July 2023.

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Methodology: Mature seeds of two black rice varieties used in this work. The seeds were dehusked manually, washed with running tap water. Then sterilized with 1% bavistin and again washed with running tap water. Seeds were surface sterilized with 0.2% $HgCl_2$ for 15 minutes. Sterilised seeds were rinsed with sterile distilled water inside laminar air flow hood. The seeds were blotting dry using autoclaved tissue paper and sterile seeds were cultured with the scutellum pointing up wards on callus induction medium fortified with different concentrations and combinations of plant growth regulators.
Results: MS medium fortified with 2,4-D was found to be better than picloram and TDZ to induce callus and the 2,4-D @ 1.00 mg/L and 2,4-D 2.00 mg/L was found to be the best concentrations. Callus health was also good when MS medium invigorated with 2,4-D.
Conclusion: An efficient embryogenic callus induction and plantlet regeneration protocol for black rice was established by using mature seeds. In callus induction studies, with different concentrations of PGR, 2,4-D, higher callus induction frequency was observed with 1.00 mg/L 2,4-D and 2,4-D @ 2 mg/L for both rice varieties.

Keywords: Black rice; callus induction; plant growth regulators; 2,4-D.

1. INTRODUCTION

"Rice (*Oryza sativa* L.) is the world most important cereal crop belongs to the family Poaceae. There are 24 species, of which 22 are wild and two are cultivated, *viz. Oryza sativa* and *Oryza glabrrima"* [1]. "In Asia, where about 90% of the world's rice production and consumption is found. It provides one-third of total dietary carbohydrate, especially in Asian countries and it is stable diet for more than three billion people, supplying 50-80% of their daily calorie intake" [2]. Traditional plant breeding is unable to keep up with the increased need for rice production, which is being driven by both population growth and climate change.

"Asian countries, like China, Japan, India, Sri Lanka, Thailand, Indonesia, Myanmar and Bangladesh cultivated black rice" [3] (Sangma and Parameshwari, 20023). "Black rice cultivars in India are comes under special rice varieties with a dark-red colour dehusk kernel, soft and aromatic when cooked. Black rice contains relatively high anthocyanin (primarily *cyaniding-3-O-glucoside and peonidin 3-O-glucoside*) in the pericarp layer which gives the dark purple color" [4,5]. "Compared to white rice, black rice has higher minerals (iron), vitamins, and bioactive substances (anthocyanin, flavonoid, and phenolic compounds). Additionally, the strong antioxidant activity found in coloured rice helps lower the risk of various chronic illnesses, including diabetes, cancer, and cardiovascular conditions" [6]. "Black rice is low in sugar but packed with healthy fibre and plant compounds that combat heart disease and cancer, according to scientists" [7].

"The culture of dehusked rice seeds is a useful method for utilising somaclonal variation. But its application is limited by many factors which influence culture efficiency, such as plant genotype" [8], the culture methods, the media [9] and the culture conditions. "The callus induction frequency and regeneration percentage were influenced by genotype, callus induction medium, regeneration medium, interaction between genotype and the two media (callus induction and regeneration) as well the interaction between the callus induction medium and regeneration medium" [10]. "Genotype and culture medium are the two principal factors that affect the end result of an *in vitro* grown culture" [10]. "The callus induction and plant regeneration in rice has been successfully obtained from different type of explant such as mature seeds, immature seed, anther, leaf and root" [11]. "Other explants such as node or shoot tip has a less callogenesis potential. Production of callus and its subsequent regeneration are the prime steps in crop plant to be manipulated by biotechnological means and to exploit somaclonal variation" [11].

The choice of the proper medium is the first step in any tissue culture experiment because it is the most important factor for the experiment's success. Thus the objectives of this investigation were to study the effect of different concentrations and combinations of plant growth regulators on callus induction in black rice.

2. MATERIALS AND METHODS

2.1 Materials

Mature seeds of two black rice varieties viz., Chakhao Amubi and Kalobhat used in this work. The genotypes were obtained from the Rice

Table 1. Different concentrations and combinations of plant growth regulators

Treatments	Concentrations and combinations of plant growth regulators
T1	MS + 2,4-D @ 1.0 mg/L
T2	MS + 2,4-D @ 2 mg/L
T3	MS + 1.0 mg/L of 2,4-D + 0.50 mg/L of NAA
T4	MS + 2.0 mg/L of 2,4-D + 0.50 mg/L of NAA
T5	MS + Picloram @ 0.50 mg/L
T6	MS + Picloram @ 1.0 mg/L
T7	MS + Dicamba@ 0.50 mg/L
T8	MS + Dicamba@ and 1.0 mg/L
T9	MS + TDZ @ 0.25 mg/L
T10	MS + TDZ @ 0.50 mg/L

Germplasm Repository as maintains by Prof. Bidhan Roy at Uttar Banga Krishi Viswavidyalay, Pundibari, Cooch Behar.

2.2 Experimental Setup

The seeds were dehusked manually, washed with running tap water for 10 minutes. Then sterilized with 1% (w/v) bavistin for 10 min and washed with running tap water. Seeds were immersed in sterile distilled water for 1 hour. The soaked seeds were surface sterilized with 0.2% (w/v) mercuric chloride for 15 minutes. Finally, the sterilised seeds were rinsed with sterile distilled water for five times inside laminar air flow hood to remove all the trace of mercuric chloride. The seeds were blotting dry using autoclaved tissue paper and sterile seeds were cultured with the scutellum pointing up wards on callus induction medium [12] fortified with different concentrations and combination of plant growth regulators (Table 1). Cultures were incubated in culture room in dark at 25 ± 2°C temperature.
After three weeks of inoculating cultures, germination (%), callus induction ability (%) and callus health were recorded.

The callus induction medium from each experiment was composed of 3% (w/v) sucrose and solidified with 0.8% (w/v) agar, the pH of the culture medium was adjusted to 5.7-5.8 before autoclaving at 121°C, 1.07 kg/ cm^2 for 15 min.

2.3 Statistical Analysis

All the tissue culture experiments of germination and callus inductionwere analysed in completely randomized design (CRD) with 3 replicates per treatment (200 explants per replication). Data were tested by using two-way analysis of variance (ANOVA) and the significant differences among means were separated by Duncan's multiple range test (DMRT) ($p \leq 0.05$) using the program GRAPES 1.0.0.

3. RESULTS AND DISCUSSION

3.1 Seed Germination (%)

Within the first week, germination of the cultured seeds began. The majority of cultures exhibited either radical development leading to root formation or plumule development leading to shoot production. Some cultures developed root and shoot growth.

Among all ten MS media combination with PGR, it was found that the MS medium fortified with 2,4-D @ 1.0 mg/L had highest germination (95.4%) followed by 2,4-D @ 2 mg/L (94.8%) and Picloram @ 0.50 mg/L (94.8%). Lowest germination percentage (91.2%) was observed when MS medium was fortified with 2.0 mg/L of 2,4-D + 0.50 mg/L of NAA. The effect of the three media (T6, T7, T8 and T10) on germination percentage was at par among both the rice cultivars (Table 2).

Both the cultivars exhibit significant variations for germination percentage among all culture media combination with PGR. The germination percentage of Chakhao Amubi ranges from 94.5% to 98.5% with an overall average 96.5%. Whereas germination percentage of Kalobhat ranges from 87.2% to 94.5% with an average 90.5% (Table 2).

3.2 Callus Induction

In certain cultured rice cultivars, the germ pore of the seed started to develop a callus after three weeks, whereas in others, the base of the beginning stalk grew and developed a callus after a month. More cultures than cultures from the germ pore of cultivated seeds demonstrated

callus development. Colour, nature, and growth of the callus differed from cultivar to cultivar and from one media to another. The majority of the calli were cream, however the colour ranged from cream to brown.

The overall beat callus induction ability (88.6%) was found in seeds cultured on MS medium invigorated with 2,4-D @ 1.0 mg/L which was followed by 2,4-D @ 2 mg/L (Table 3, Fig. 1). Susanto et al. [13] also reported higher callus induction ability in Indonesian black rice when MS medium was supplemented with 2,4-D @ 2 mg/L. It was also found the cultured seeds on medium different concentrations of dicamba and TDZ had no effect on callus induction. So, those combination cultures were not suitable for callus induction of selected black rice varieties (Table 3). The callus induction ability of Chakhao Amubi ranges from 0.0% to 91.1% to with an average 27.6%. In case of Kalobhat the callus induction ability ranges from 0.0% to 86.0% with a grand mean 27.1% (Table 3).

Cultivar plays important role in callus formation. Chakha Amubi showed better callusing response than Kalobhat (Table 3). Callus development is genotype-dependent, just like any other tissue culture response, and some genotypes performing better than others [14,15]. Yang et al. [16] had observed higher callus induction in Japonica rice varieties as compared to Indica varieties. Further, the modern high yielding varieties had higher callus induction ability than the traditional varieties [14,15]. Thus, high callus induction response in traditional black rice of North-eastern India was novel finding in this endeavour. MS medium fortified with 2,4-D @ 1.0 mg/L may be recommended for callus induction for black rice. This results were also corroborative with the findings of Rahman et al. [17] for black rice.

Table 2. Germination (%) of cultured seeds of rice cultivars on different media

Culture Media	Chakhao Amubi	Kalobhat	Mean value
MS + 2,4-D @ 1.0 mg/L	96.7^{abc}	94.2^{de}	95.4^{a}
MS + 2,4-D @ 2 mg/L	95.2^{bcd}	94.5^{cde}	94.8^{ab}
MS + 1.0 mg/L of 2,4-D + 0.50 mg/L of NAA	94.7^{bcde}	89.7^{gh}	92.2^{cd}
MS + 2.0 mg/L of 2,4-D + 0.50 mg/L of NAA	94.5^{cde}	87.8^{hi}	91.2^{d}
MS + Picloram @ 0.50 mg/L	96.8^{abc}	92.7^{ef}	94.8^{ab}
MS + Picloram @ 1.0 mg/L	97.7^{a}	90.0^{gh}	93.8^{abc}
MS + Dicamba@ 0.50 mg/L	98.5^{a}	88.8^{ghi}	93.7^{abc}
MS + Dicamba@ and 1.0 mg/L	97.0^{ab}	90.3^{fg}	93.7^{abc}
MS + TDZ @ 0.25 mg/L	97.0 ^{ab}	87.2^{i}	92.1^{cd}
MS + TDZ @ 0.50 mg/L	97.0^{ab}	90.0^{gh}	93.5^{bc}
Range	94.5-98.5	87.2-94.5	91.2-95.4
Mean value	96.5	90.5	93.5

Values followed by the same letter in row are not significantly different using Duncan's Multiple Range Test (DMRT) at p ≤ 0.05

Table 3. Callus induction ability (%) of cultured seeds of rice cultivars on different media

Culture Media	Chakhao Amubi	Kalobhat	Mean value
MS + 2,4-D @ 1.0 mg/L	91.1^{a}	86.0^{b}	88.6^{a}
MS + 2,4-D @ 2 mg/L	79.9^{c}	82.4^{ab}	81.1^{b}
MS + 1.0 mg/L of 2,4-D + 0.50 mg/L of NAA	57.2^{c}	54.1^{c}	55.7^{c}
MS + 2.0 mg/L of 2,4-D + 0.50 mg/L of NAA	48.0^{d}	48.2^{d}	48.1^{d}
MS + Picloram @ 0.50 mg/L	0.0^{e}	0.0^{e}	0.0^{e}
MS + Picloram @ 1.0 mg/L	0.0^{e}	0.0^{e}	0.0^{e}
MS + Dicamba@ 0.50 mg/L	0.0^{e}	0.0^{e}	0.0^{e}
MS + Dicamba@ and 1.0 mg/L	0.2^{e}	0.0^{e}	0.1^{e}
MS + TDZ @ 0.25 mg/L	0.0^{e}	0.0^{e}	0.0^{e}
MS + TDZ @ 0.50 mg/L	0.0^{e}	0.0^{e}	0.0^{e}
Range	0.0-91.1	0.0-86.0	48.1-88.6
Mean value	27.6	27.1	27.4

Values followed by the same letter in row are not significantly different using Duncan's Multiple Range Test (DMRT) at p ≤ 0.05

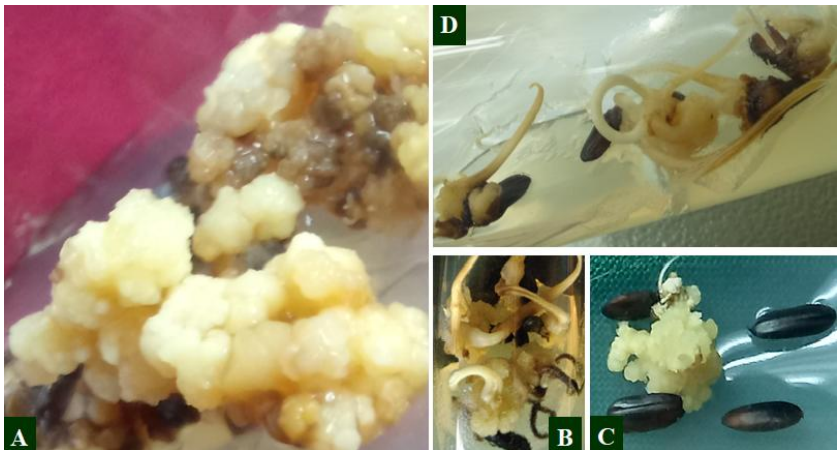

Fig. 1. A- Callus produced by Chakhao Amubi on CIM MS + 2,4-D @ 1.0 mg/L; B- MS + 2,4-D @ 2 mg/L; C- MS + Yeast extract @ 200 mg/L; D- MS + maltose @ 6%

Table 4. Health of callus formed on cultured seeds of rice cultivars

Culture Media	Chakhao Amubi	Kalobhat
MS + 2,4-D @ 1.0 mg/L	1.0	1.0
MS + 2,4-D @ 2 mg/L	1.0	1.0
MS + 1.0 mg/L of 2,4-D + 0.50 mg/L of NAA	1.7	2.3
MS + 2.0 mg/L of 2,4-D + 0.50 mg/L of NAA	2.3	2.3

Fig. 2. A- Callus produced by Kalobhat on CIM MS + 2,4-D @ 1.0 mg/L; B- MS + 2,4-D @ 2 mg/L; C- MS + Yeast extract @ 200 mg/L; D- MS + maltose @ 6%

3.3 Callus Health

The health of callus recorded from different culture media combinations of first experiment was observed and the rating of 1 to 9 given as per the health of callus. It was found that the callus obtained from MS medium supplemented with 2,4-D @ 1.0 mg/L and 2,4-D @ 2 mg/L was excellent than the culture combination containing 1.0 mg/L of 2,4-D + 0.50 mg/L of NAA, 2.0 mg/L of 2,4-D + 0.50 mg/L of NAA (Table 4, Fig. 2).

4. CONCLUSION

In this work, two types of black rice (Oryza sativa L.) were chosen to examine in vitro callus induction. Plant growth regulators were investigated for promoting callus induction in selected rice types when combined with basal MS medium. Using mature seeds, a successful technique for the development of embryogenic calluses and plantlet regeneration for black rice was developed. In callus induction studies, with different concentrations of PGR, 2,4-D, higher callus induction frequency was observed with 1.00 mg/L 2,4-D and 2,4-D @ 2 mg/L for both rice varieties.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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