Selection of purification and formation of double haploid Toraja endemic black rice through anther culture

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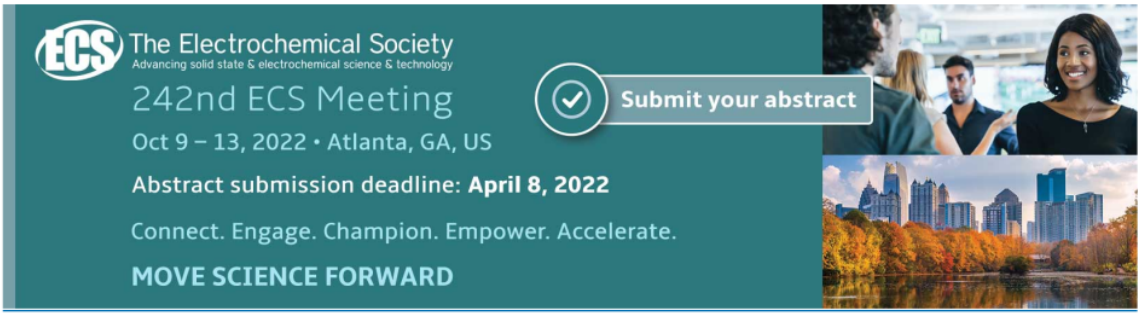

Selection of purification and formation of double haploid Toraja endemic black rice through anther culture

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Abstract. To maximize the potential of Ko'bo, plant breeding was involved to produce the identic color of rice grains, which is full black. Anther culture is a method that is considered effective in plant breeding. This study aimed to find the most suitable combination of growth regulators in the calli induction of Ko'bo rice anther and observe the calli growth response in a regeneration medium. Calli induction experiment used N6 medium with a combination of auxin (2,4-D 1-3 mg L^{-1}) and cytokinin (kinetin 0.5-1 mg L^{-1} and BAP 0.5-1 mg L^{-1}). Calli regenerated in MS medium, added with 0.5 mg NAA L^{-1} + kinetin 0.5 mg L^{-1}+ BAP 1.5 mg L^{-1}. The experiment was arranged in a Completely Randomized Design. The experimental unit was a petri dish, containing ± 250 anthers. The highest calli induction was 13.33%, obtained from a combination of 2,4-D 3.0 mg L^{-1} + kinetin 1.0 mg L^{-1}. All regenerated plantlets were albino.

1. Introduction

In North Toraja Regency, Indonesia, black rice is one of the local rice used for generations as part of the traditions and culture of the community. The use of black rice in traditional ritual ceremonial culture seems to be an important part of its preservation efforts. Black rice from the region of North Toraja Regency has a unique phenotype character. The 'black' color obtained by dark purple color, due to its high anthocyanin content. Anthocyanin is a water-soluble pigment, which has antioxidant activity. Black rice is known to be effective to increase the body's resistance to disease, repair damage of liver cells, prevent impaired kidney function, prevent cancer/tumors, delay aging, as antioxidants, clean cholesterol in the blood, and prevent anemia [1,2].

Toraja local black rice has a very low level of purity. Initial studies have been carried out, showing that in one panicle there are a variety of different seed colors. If rice were cut horizontally, based on the color inside the rice, it can be grouped into three groups, which are fully black, medium black, and thin black. To optimize Toraja black rice as one of the potential sources of germplasm, the first step that needs to be done is to purify black rice varieties. This purification begins with the selection of the

color of rice, which is black rice that is completely black inside and outside. Then continued with anther culture to produce double haploid plants. The existence of this technique significantly affects the improvement and efficiency of plant breeding and seedling programs. Thus, anther culture is expected to be the basis for the formation of new superior genotypes of high-quality black rice. The application of anther culture in rice breeding is reported to have succeeded in obtaining superior varieties in rice-producing countries including in China and Korea [3,4]. On several other reports, anther culture applied to obtain pure lines as a selection material to speed up the process of assembling varieties [5-8], and anther culture applied to obtain new superior genotypes [9].

To improve the response of indica rice anther calli formation, Dwimahyani et al. [10] obtained the best result from 2,4-D and kinetin for calli induction on N6 media combined with microelements of MS media. Rout [11] obtained a high percentage of successful calli induction on the N6 medium supplemented with 2,4-D and BAP. The study stated that a higher percentage of regenerated green shoot obtained from calli induced in N6 medium and transferred into the MS regeneration medium.

2. Materials and method

2.1. Plant materials

The study was conducted at the Laboratory of Biosciences and Plant Reproductive Biotechnology in Teaching Industry and Screen house of Agriculture Faculty, Universitas Hasanuddin. Toraja black rice seeds were obtained from the selection results of the previous conventional breeding study. The black rice was grown following standard agronomic practices.

2.2. Anther pre-treatment.

Panicles from plants were harvested between 4 pm to 5 pm on sunny days. Panicles were harvested when the distance between the collar of flag leaf and penultimate leaf was about 7-12 cm. Panicles were wrapped in aluminum foil with a wet towel, stored for 8-10 days in cold temperatures at 5 °C.

2.3. Anther culture

The selected panicles were sterilized with 20% NaHClO for 20 minutes and rinsed with sterile water for three times. Sterile spikelets were cut 1/3 part at the base using scissors. The spikelets were knocked on the edge of the Petri dishes until the anther comes out and falls onto the medium. Petri dish contained 25 mL of agar solidified N6 medium supplemented with combination of 2,4-D (1-3 mg L^{-1}), Kinetin (0, 0.5, and 1.0 mg L^{-1}), and BAP (0, 0.5, and 1.0 mg L^{-1}). Each petri dish contained anther from 25 spikelets. One petri dish constituted one replicate and an average of 5 replicates were cultured for each treatment. The culture was incubated in a dark room at 25± 2°C for calli induction.

2.4. Plant regeneration

Calli with 1-2 mm diameter were transferred into regeneration medium, agar solidified Murashige and Skoog (MS) supplemented with NAA 0.5 mg L^{-1}, Kinetin 0.5 mg L^{-1} and BAP 1,5 mg L^{-1}. The cultures were given a 16-hour photoperiod. The regenerated shoots were transferred to hormone-free MS medium for rooting.

2.5. Data analysis

The parameters observed were calli induction efficiency and regeneration of various calli induction media, and the best calli induction media that produced calli and non-albino rice plants. Path coefficient analysis was performed using SPSS version 25. The observational data obtained were analysed invariance and if there was a significant effect on food treatment continued using the Duncan's Multiple Range Test (DMRT) test (p<0.05).

3. Results and discussion

Toraja rice, Kobo rice, belongs to the indica rice subspecies. In various studies, indica rice known for being recalcitrant which can be observed by a very low number of formed calli from anther, low ability to generate and the high percentage of regenerated albino plants [12,13]. In this study, calli were found in all treatment mediums with a variety of percentages. The following are the results of calli induction experiments on various treatments of growth regulators as listed in table 1. Table 1 showed the largest percentage of calli was obtained from a combination of 2,4-D 3.0 mg L^{-1} Kinetin 1.0 mg L^{-1}, which is 13.33%. Meanwhile, the lowest percentage obtained from combination 2,4-D 2.0 mg L^{-1} + Kinetin 1.0 mg L^{-1}, which is 0.27%.

Table 1. Percentage of calli formation from rice anther in various growth regulators

Treatment (mg L^{-1})	Symbol	Number of formed calli	Percentage of formed calli (%)
1.0 2,4-D + 0.5 Kin	H1	19	2.53^{ab}
1.0 2,4-D + 1.0 Kin	H2	13	1.73^{a}
1.0 2,4-D + 0.5 BAP	H3	7	0.93^{bc}
1.0 2,4-D + 1.0 BAP	H4	3	0.40^{a}
2.0 2,4-D + 0.5 Kin	H5	7	0.93^{a}
2.0 2,4-D + 1.0 Kin	H6	2	0.27^{a}
2.0 2,4-D + 0.5 BAP	H7	14	1.87^{a}
2.0 2,4-D + 1.0 BAP	H8	4	0.53^{a}
3.0 2,4-D + 0.5 Kin	H9	24	3.20^{ab}
3.0 2,4-D + 1.0 Kin	H10	100	13.33^{c}
3.0 2,4-D + 0.5 BAP	H11	24	3.20^{ab}
3.0 2,4-D + 1.0 BAP	H12	76	10.13^{c}

Numbers having the same letter within a column are not significantly different from the DMRT test at $p<0.05$.

Anthers began to show the response of the formation of calli in the third week. The initial sign that can be observed with bare eyes is a small white dot that appears on one side of the anther. The dot is microspores cells that divide and gradually form a microcolony which becomes the calli [10]. The calli mass enlarges to form clumps that are white to yellowish. The texture found among calli also varies. Calli from 8 treatments (H1, H2, H5, H6, H7, H8, H9, H10) were crumb, while the rest were compact. The duration of the calli formation also varies, from 3 weeks to 10 weeks. The calli still appeared even after the observation ended. Dwimahyani et al [10] also found that the formation of calli even continued at 12 weeks after anther planting.

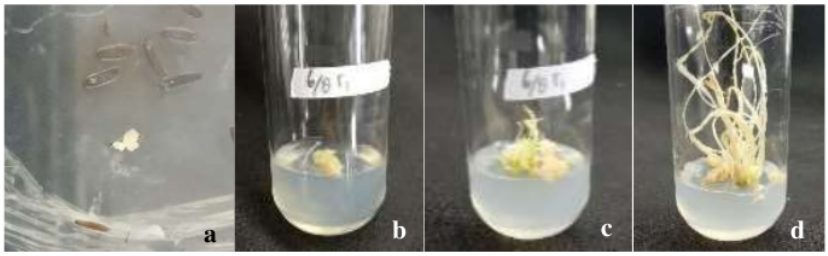

Figure 1. (a) Calli on calli induction media (b) Calli transferred to regeneration media (c) Calli differentiating to form leaves (d) albino plants

According to Fehr [14], there are main factors that need to be considered in anther culture namely source plants, stage of anther development and the culture environment. The source plant directly related to the pollen genotype has a significant effect on the percentage of rice anther calli formation. The anther culture is specific to each cultivars, so the treatment of different rice cultivars will give different results. In a study conducted by Herath et al [15], the percentage of calli formation from japonica rice anther was 12.3-70.7%. Whereas calli induction on indica rice anther only reached 0.3-0.7%. Likewise, Sripichitt et al. [16] found calli formed from japonica rice calli culture was 13-17% while calli formed from indica rice anther was only 1-4.4%. The success of calli induction in this study found to be higher, which is 13.33%, obtained from a combination of 2,4-D 3.0 mg L^{-1} +Kinetin 1.0 mg L^{-1}.

The result was similarly found from a combination of 2,4-D 3.0 mg L^{-1} + BAP 1.0 mg L^{-1}, produced 10.13% of calli formation. The higher percentage of calli induction can be caused by a higher dose of 2,4-D. This proves the treatment of auxin with higher doses can significantly improve the efficiency of calli growth.

In each treatment, the number of calli that appeared in each petri dish was always different although receive the same treatment. Although the medium used is the same, the need for special treatment of phytohormone is very specific depending on plant genotype. The number of calli produced per anther varies greatly, even if the anther originates from the same panicle, is placed on the same calli induction media, and is cultured under the same environmental conditions [12].

The determination of calli transferred into a regeneration medium when it reaches its ideal size, which is 1-2 mm in diameter. The appropriate size of the calli diameter is based on various studies. Studies that report that a callus that is less than 1 mm in size will find it difficult to regenerate or die. Purmaningsih [17] reported that indica rice calli with a diameter bigger than 0.9 mm able to regenerate into plantlets as much as 50% success. Calli transfer must be carried out on time, calli that are more than two months old in calli induction media do not have the ability to differentiate [10]. Within two to three weeks, on a few calli on regeneration media appear small pale light green spots, most of the calli shows no green spots at all. In the third to fourth week, the calli begin to differentiate to form pale leaves and or roots.

Table 2. The response of shoot and root regeneration in MS medium from different calli induction medium.

Treatment	Green shoot	Albino plantlet	Formation of roots only
1.0 2,4-D + 0.5 Kin	0	3	0
1.0 2,4-D + 1.0 Kin	0	1	1
1.0 2,4-D + 0.5 BAP	0	1	0
1.0 2,4-D + 1.0 BAP	0	1	0
2.0 2,4-D + 0.5 Kin	0	2	1
2.0 2,4-D + 1.0 Kin	0	1	0
2.0 2,4-D + 0.5 BAP	0	1	0
2.0 2,4-D + 1.0 BAP	0	2	0
3.0 2,4-D + 0.5 Kin	0	4	0
3.0 2,4-D + 1.0 Kin	0	8	2
3.0 2,4-D + 0.5 BAP	0	3	1
3.0 2,4-D + 1.0 BAP	0	11	3

Plantlet regeneration varies greatly from 8.33% to 100% of the number of calli transferred to the regeneration media. The highest percentage of regeneration is obtained in treatments that produce fewer calli. Albino plants were obtained in all treatments with a percentage of 100% (Table 2). Albinism is a quite widespread phenomenon in the indica activity of indica rice culture. Albino plants

are undesirable because they cannot be acclimatized due to the absence of chlorophyll in these plants, so they will not survive. This also happened in the study of Mandel and Bandyopadyay [12], a genotype produced 100% albino plants, while other 16 genotypes of rice planted on the same medium showed the formation of albino plants from 0% to > 50%.

Table 2 shows the calli response to MS regeneration media. None of the calli produced any green plantlet. Although a combination of 2,4-D 3.0 mg L^{-1} + kinetin 1.0 mg L^{-1} produced the highest percentage of calli, the regeneration rate was very low. A similar situation was reported by Kaushal et al. [18], that sometimes there are genotypes that show a high percentage of calli induction but their regenerative ability tends to below, and vice versa.

In addition to growth-regulating factors, genotype, and calli diameter as discussed above, the success of anther culture can be supported by a variety of other factors. These factors include the choice of calli induction and regeneration medium [16,18-19], duration of low temperature pre-treatment [15,20], source plant growth environment [21], spikelet location in panicle [22], source nitrogen [23], and carbon source [20]. The low success of indica rice culture, the high albino plants and the low percentage of green plants, are a major challenge that needs to be resolved through chemical manipulation and the physical environment combined with other innovative methods. The use of liquid culture media, interbreeding or changing the type of carbohydrate are alternatives that are worth a try [12].

4. Conclusions

The highest success of calli induction in Ko'bo rice was 13.33% obtained from a combination of growth regulator 2,4-D 3.0 mg L^{-1} + kinetin 1.0 mg L^{-1}. All regenerated plantlets are albino.

Acknowledgment

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