

Effect of cold pre-treatment of panicle on induction and regeneration of callus from anther culture of seven rice genotypes

Yoanita Fadlilah Iriani^{1,*}, Dian Catur Prayantini², Retno Mastuti¹, Nunung Harijati¹

¹Department of Biology, Mathematics and Natural Sciences Faculty, Universitas Brawijaya, Malang 65145, Indonesia

²Department of Biotechnology, PT. BISI International, Tbk. Jl. HOS Cokroaminoto No. 72A, Mulyoasri, Tulungrejo, Pare, Kediri 64212, Indonesia

Abstract

Anther culture is an in vitro culture technique to produce double haploid plants with homozygous traits, which can be used to make new varieties of plants more quickly. The success of anther culture was influenced by several factors, including plant genotype, culture media composition, culture environment conditions (temperature and light), and pretreatment of anther before culturing. Cold temperature pretreatment was an important factor to support the success of anther culture. The purpose of this study was to determine the effect of cold pretreatment on panicle induction and callus regeneration from anther culture of seven Indica rice genotypes (*Oryza sativa* L.). Panicles used as explants were harvested, then pretreated with cold temperatures of 4°C and 8°C for 8 days. The anther was cultured on callus induction media consisting of 3,21 g/L B5 Gamborg, 50 g/L maltose, 2 g/L Gelrite, 2,5 mg/L NAA, 1 mg/L Kinetin, with 2 mg/L 2,4-D. The formation of callus is observed to occur at a period of four to six weeks following the culture process. The regeneration of callus is facilitated by a medium specifically formulated for this purpose, comprising MS medium along with additional components, 30 g/L sucrose, 2 g/L Gelrite, 1 mg/L BAP, 1 mg/L Kinetin, 1 mg/L NAA, pH 5,8. Two weeks after the callus was regenerated, the percentage of green callus and browning callus formed was calculated. The 8°C cold pretreatment produced the highest green callus, while the highest browning callus was obtained in the 4°C cold pretreatment. The total callus formed was positively correlated with the total green regenerants produced. Cold pretreatment at 8°C for 8 days can increase the responsiveness of anther culture by providing the best results for induction and regeneration in the seven plant genotypes used in this study.

Keywords: anther culture, callus, cold temperature, pretreatment, rice.

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Introduction

Rice (*Oryza sativa* L.) is the staple food source of most of the world's population, including Indonesia. The demand for food, especially rice, continues to increase along with the increase in population. (Fuqara & Tanjung, 2023). One of the efforts to meet the increasing demand for food was the use of superior varieties with high yield (Arianti et al., 2020). The assembly of superior rice varieties can be achieved both through conventional breeding and through biotechnology biotechnological techniques, such as the anther culture approach (Dewi & Purwoko, 2016).

The anther culture method is generally applied to produce homozygous double haploid (DH) pure strains. The advantage of this method lies in its ability to produce pure strains in a relatively short time, which is achieved through the callus induction process. (Carsono et al., 2022). However, anther culture has several constraints. These include low success rates of green plant regeneration, the emergence of albino plants, limited genotype response, and ploidy variation in the resulting plants (Safitri et al., 2010). Factors that affect the success of anther culture are plant genotypes, media composition, anther pretreatment before culture, microspore development phase, environmental conditions of donor plants, and panicle collection time (Safitri et al., 2010). In addition to plant genotypes having different anther culture responses (Safitri et al., 2010), the ability of each

genotype to produce callus in rice anther culture is also different (Herawati et al., 2008). Besides genotype, pretreatment temperature showed a significant effect on green plant induction and regeneration (Gunarsih et al., 2016). Cold temperature is used to homogenize pollen development, maximizing the yield of uninucleate stadia pollen for further use (Herawati et al., 2008). Cold pretreatment of rice anther culture has a significant effect on androgenic response (Mishra & Rao, 2016). Androgenic response is the ability of the anther to produce haploid or spontaneous double haploid plants (DH) (Mayakaduwa & Silva, 2023). This study aimed to evaluate the effect of cold temperature pretreatment of panicles before anther culture on the ability to induce and regenerate callus from anther culture of seven Indica rice genotypes. The aim of using the anther culture technique to develop seven indica rice genotypes was to accelerate obtaining homozygous plants that would be used as F1 elders.

Methods

Plant materials

Donor plants of rice (*Oryza sativa* L.) were obtained from the seed collection of the Rice Breeding Team of PT BISI International, Tbk, Farm Kambangan, Kediri, East Java. The donor plants are elite lines from Indica rice, the codes are as follows: RCKJ 01, RCKJ 02, RCKJ 03, RCKJ 04, RCKJ 05, RCKJ 06, and RCKJ 07. Seven donor plants were planted and maintained until they reached the generative stage, then panicles were taken as explants for anther culture. Panicles were selected and harvested based on the size of the last leaf spacing and flag leaves that were

*Corresponding Author:
Yoanita Fadlilah Iriani
Department of Biology, Mathematics and Natural Sciences Faculty,
Universitas Brawijaya, Malang 65145, Indonesia
E-mail: yoanita204@gmail.com

in the late uninucleate to early binucleate developmental phase.

Panicle pre-treatment

The harvested panicles were then wrapped in wet tissue and rolled into plastic (Fig. 1A-B). Wrapped panicles then incubated in the dark at 4°C and 8°C for 8 days.

Callus induction and regeneration

Explant selection was done by selecting spikelets with anther position not exceeding half the spikelet length. Explants were sterilized using 70% alcohol for 2 min, 40% NaOCl for 15 min, and finally rinsed 3x with sterile distilled water. Sterilized spikelets were cut at 1/3 of the base to remove the anther. The anther was cultured in callus induction media, and incubated in the dark at 25°C. Each treatment was repeated four times (four petri). Each petri contained 125 rice anther. After 4-6 weeks of culture, callus formed. Then the callus was regenerated and incubated in the light with 16 hours light and 8 hours dark at 25°C. The parameters observed included the total number of callus formed, the percentage of green callus, the percentage of browning callus, and the total number of green plantlets produced. Total number of callus obtained from total callus formed anthers, and it should be noted that one anther can produce one to tens, even hundreds of callus, because one anther contains a large number of microspores, each microspore has the possibility to develop into one callus. Callus measuring 1-2 mm were separated from the anther slowly, without injuring the callus, and regenerated. Calculation of the number of callus was done during regeneration, by transferring callus one by one from callus induction media to regeneration media.

$$\frac{\Sigma \text{ number of callus}}{\Sigma \text{ Callus formed from 125 anthers at 28 days after induction (Dewi et al. 2006)}}$$

$$\text{Green callus (\%)} = \frac{\Sigma \text{ Green callus}}{\Sigma \text{ number of callus}} \times 100\%$$

$$\text{Browning callus (\%)} = \frac{\Sigma \text{ Browning callus}}{\Sigma \text{ number of callus}} \times 100\%$$

$$\text{Green plantlets (\%)} = \frac{\Sigma \text{ Green plantlets}}{\Sigma \text{ number of callus}} \times 100\%$$



Figure 1. (A) Harvested panicles that will be pretreated with cold temperature; (B) Panicles that have been wrapped in wet tissue and wrapped in plastic; (C) Position of anther that does not exceed half the length of the grain

Results

Four weeks after culture, callus will form and come out of the anther explants (Fig. 2A). Cold temperature pretreatment of panicle explants to be cultured affects the results of callus induction and regeneration from rice anther culture. The formation of the callus results in the subsequent development of two distinct types of callus, green callus and browning callus. Green callus possesses the capacity for regeneration, while browning callus lacks this potential. Green callus will grow into green plantlets on regeneration media (Fig. 2B). Green plantlets will then grow into first-generation double haploid candidate plants (DH0) (Fig. 2C). The 8°C cold pretreatment was able to produce higher total callus than the 4°C cold pretreatment in all genotypes used (Fig. 3A). In genotype RCKJ 03, the total callus obtained with 8°C cold pretreatment increased up to 36 times compared to 4°C cold pretreatment, from two callus to 73 callus (Fig. 3A).

In addition to affecting callus formation, cold temperature pretreatment was also able to affect the formation of green callus and browning callus in all genotypes used (Fig. 3B and 3C). Green callus is a callus with regeneration potential, while browning callus is defined as a type of tissue that has undergone a process of chemical alteration, resulting in the secretion of phenol substances that give it a brown pigmentation. This alteration renders the tissue incapable of undergoing regeneration. The 4°C cold pretreatment produced 0.00-4.75% green callus, while the 8°C cold pretreatment produced 21.34-42.09% (Fig. 3B). This shows that 4°C cold pretreatment is not enough to produce green callus, instead it produces more browning callus in the seven genotypes used in this study (Fig. 3C).

The total green plantlets produced by 8°C pretreatment are more when compared to 4°C pretreatment (Fig. 3D). Each genotype produced green plantlets with various amounts. The RCKJ 04 genotype produced the highest number of green plantlets compared to the other six genotypes, with 62 green plantlets (Fig. 4A-B).



Figure 2. (A) Callus formed from anther explants; (B) Green callus; and (C) Green plantlets.

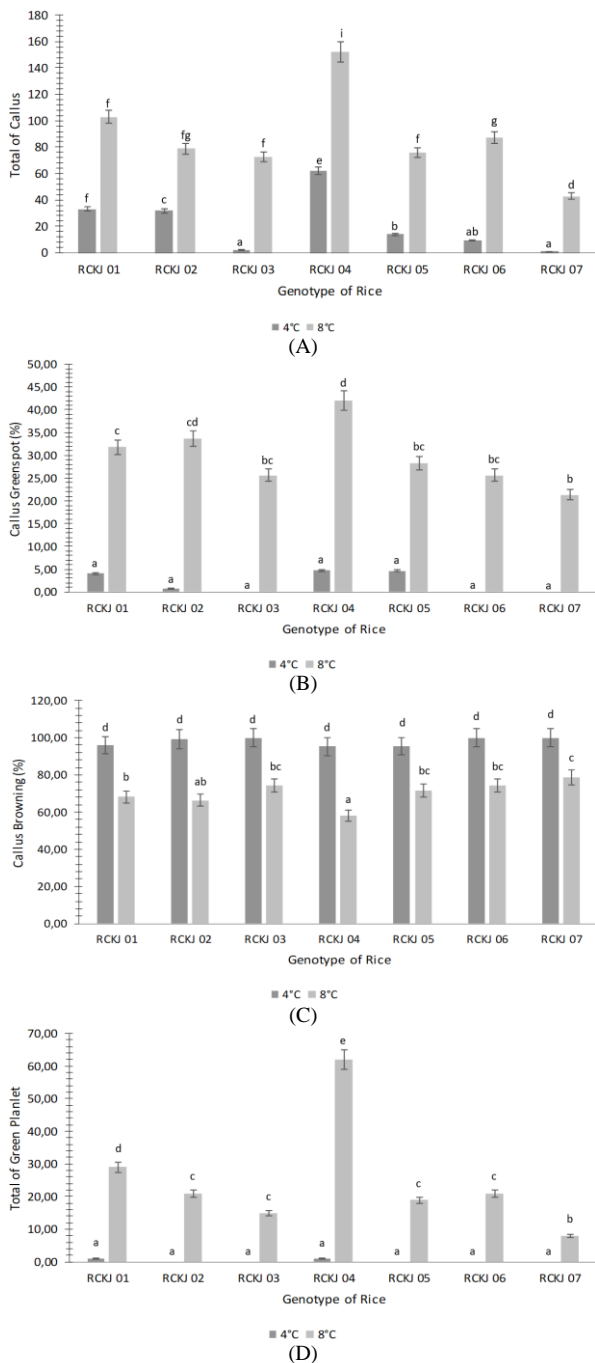


Figure 3. Effect of cold pretreatment on total callus formed (A); percentage of green callus formation (B); browning callus (C); and total green plantlets (D) formed in seven rice genotypes. The same letter in each parameter indicates not significantly different in the Tukey HSD test with a real level of 5% ($p < 0.05$)

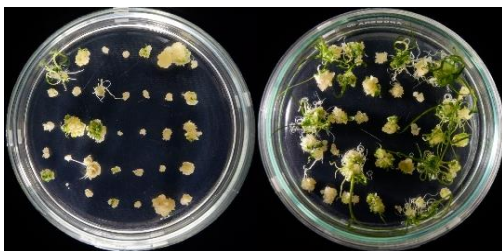


Figure 4. Green plantlets of RCKJ 04 with 4°C for 8 days cold pretreatment (A); green plantlets of RCKJ 04 with 8°C for 8 days cold pretreatment (B).

Discussion

The anther culture technique is a successful method used to obtain variation either as material for breeders or as productive material to be used for variety assembly (Na et al., 2011). Various factors such as genotype, growing environment conditions, microspore development stage, pre-treatment, culture medium, regeneration medium, growth hormones, and various chemicals have direct effects on anther culture response (Islam et al., 2023).

An anther exposed to various pretreatments (cold temperature, heat temperature, osmotic stress, sugar starvation, gamma irradiation, and chemical treatment) before culture is reported to induce androgenesis (Tripathy et al., 2019). Pretreatment also inhibits callus formation from somatic cells (anther wall and tapetum). However, the type and duration of pretreatment varies among rice species and varieties (Mishra & Rao, 2016).

Cold treatment helps to slow down the aging process of the anther wall, thus maintaining anther health for longer. By delaying senescence, it also enhances microspore division, which is an important step in the development of haploid cells. Cold pretreatment facilitates nutrient diffusion through the anther wall. This is important for promoting sporophytic cell division, which is a crucial step in the process of plant formation from the anther. This process indicates that pollen development is inhibited, which can lead to the formation of haploids through callus formation (Sharmela et al., 2023).

Under normal conditions, microspores will develop into mature male gametophytes (i.e. functional pollen). However, cold treatment halts this natural process, preventing the microspores from continuing their gametophyte development. The cold treatment alters the developmental pathway of the microspores so that they begin to divide to form a callus (Kaushal et al., 2014). Callus produced from anther culture has the potential to regenerate into green callus, however some are unable to regenerate and instead undergo browning. Browning is a character of the appearance of brown or black color that often inhibits the growth of callus (Fauzy et al., 2016). Browning is caused by the presence of phenolic compounds activated by the enzyme polyphenol oxidase (PPO) and occurs when explants are wounded in the initiation phase (Setyawati et al., 2019). Cold stress increases the production of phenolic and flavonoid compounds and their subsequent incorporation into the cell wall as either suberin or lignin (Isah, 2019). Browning results in decreased efficiency in tissue culture, which if not handled properly can lead to decreased regenerative ability, decreased callus growth, and inhibition of shoot growth (Permadi et al., 2023).

The 8°C cold pretreatment was able to produce higher total callus, percentage value of green callus, and percentage value of green plantlets than the 4°C cold pretreatment in all genotypes used. The 8°C cold pretreatment on panicles provided sufficient stress for rice callus induction and regeneration, while 4°C cold pretreatment resulted in a decrease in the number of callus

capable of being induced and affected callus regeneration ability.

Cold pre-treatment of rice panicles before anther culture serves to induce cell division and dedifferentiation of microspores towards the sporophytic pathway (producing callus and plants instead of pollen). However, too low a temperature such as 4°C may provide too extreme cold stress, causing decreased metabolic activity, membrane damage, or enzymatic inhibition, which may inhibit microspore viability. In contrast, a temperature of 8°C may be low enough to induce the sporophytic pathway through mild stress signaling, without causing physiological damage. At this temperature, genes related to cold response and cell defense may be activated, such as CBF/DREB genes, HSPs (heat shock proteins), or other anti-stress proteins, which protect the microspores during the treatment period. The result is an increased callus response rate or plant regeneration in anther culture.

Cold pre-treatment rice anthers to 7°C for three days significantly enhanced anther culture response compared to those without pretreatment (Chaleff & Stolarz, 1981). Similarly, (Cho & Zapata, 1990) found that an 8°C cold shock applied for eight days yielded the most favorable results. In another study, the importance of cold pretreatment for successful androgenesis in indica rice, identifying 10°C for ten days as the most effective condition for optimal culture performance (Bhojwani, 1973). Cold pretreatment of panicles at 5°C is highly effective in rice (Croughan & Chu, 1991); however, extended exposure of immature inflorescences to cold can reduce their induction potential. It has been reported that a critical condition for successful cold pretreatment is maintaining panicles at 10°C for 28 days prior to microspore isolation (Khatun et al., 2012).

The percentage value of green callus is opposite to the percentage of browning callus formed in the seven genotypes used. The higher the green callus percentage value, the lower the percentage of browning callus formed. This can be observed in all genotypes, one of which is RCKJ 04 which produces the highest green callus with 8°C cold temperature pretreatment (42.09%). The lowest percentage of browning callus among the seven genotypes used was also produced by RCKJ 04 (57.91%), where the percentage of browning callus from all genotypes ranged from 57.91% to 100%.

The percentage of green callus affects the total green plantlets that will be formed. Green callus has a high potential for regeneration into green plantlets. In the study, one callus can produce more than three green plantlets (Dewi et al., 2016). Green dots appear on the callus surface as an indicator of the beginning of differentiation. Green dots are areas where cells begin to form active chloroplasts, indicating the beginning of photosynthetic activity. Green dots extend over the entire surface of the callus turning into green callus. Green callus is a callus with high physiological activity and thus shows great viability and regeneration potential. Green callus leads to organogenesis, small shoots emerge from the callus, then axial growth and formation of small leaves known as green plantlets occur. Callus that shows a tendency to be

green is then followed by shoot organogenesis (Prihatmanti & Mattjik, 2004). In this study, the highest total green plantlets were obtained from genotype RCKJ 04 with 8°C cold pretreatment, which was 62 green plantlets. The 4°C cold pre-treatment produced a lower total green plantlets, between 0-1 plantlets. Meanwhile, 8°C cold pre-treatment produced a total of 8-62 green plantlets (Fig. 2D).

Genotype, culture pretreatment conditions, and culture media affect the ability of microspores to form callus, and their regeneration to form shoots and plantlets (Nurhasanah et al., 2016). The high efficiency of anther culture is reflected in the high number of regenerated green plantlets (Yan et al., 1996). Cold temperature treatment of rice anther culture has a significant effect on various aspects of callus and plantlet development. In particular, pretreatment temperature affects the total callus formed, the percentage of green callus (which has the potential to develop into green plantlets), the percentage of browning callus (which oxidizes and dies), and the total green plantlets produced. Therefore, determining the right pretreatment temperature is very important to optimize the results of rice anther culture. The optimal temperature may vary depending on the rice genotype and other culture conditions. Further research is needed to determine the best pretreatment temperature for each rice variety, so as to increase the efficiency of rice production through tissue culture techniques. Improved and faster plantlet regeneration allows for the production of more double haploid (DH) lines in a shorter time. DH lines are instantly homozygous, which significantly shortens the breeding cycle compared to conventional methods that require multiple generations. A larger number of DH lines means breeders can screen more genotypes quickly for desirable traits. Anther culture allows early-stage selection at the microspore level, before full plant development. Efficient regeneration means more individuals can be evaluated *in vitro*, reducing reliance on time- and resource-consuming field trials. Early screening under controlled conditions (e.g., for stress tolerance or herbicide resistance) makes the selection process faster and more precise. High regeneration efficiency enables the parallel testing of diverse genetic combinations, increasing the diversity of selectable lines, this supports data-driven decisions in breeding.

Conclusion

Induction and regeneration of callus from rice anther culture are influenced by cold temperature pretreatment before culture. Cold pretreatment at 8°C for 8 days can increase the responsiveness of anther culture by giving the best results for induction and regeneration in the seven plant genotypes used in this study.

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