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Full Length Article

Improving Anther Regeneration in Two Transgenic Rice (*Oryza sativa*) Genotypes using Cold Pre-Treatment

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Abstract

Anther culture is one of the *in vitro* culture techniques that can be used to obtain genetic diversity of plants. Pure line can be obtained rapidly from the pollen that establishes the plantlets. Induction of callus from the anther is influenced by the ability of anther to change from the gametofitic to sporofit pathway, and cold pre-culture have an important role in the changes of these pathways. This research aims to determine the optimum temperature of cold pre-treatment in the anther of two transgenic rice genotypes on the increasing callus induction and plant regeneration. Anther from two genotypes of transgenic rice overexpressed protein kinase *OsWee1* gene was used in this study. The first factor was transgenic rice genotypes, and the second was cold pre-culture at 4, 7 and 15°C. The results showed that two of transgenic rice genotypes responded differently to the cold pre-treatment. Cold pre-treatment at 7°C provided significant variation in callus induction and plant regeneration of rice, evident with decreasing percentage of anther browning, rapidity in inducing callus formation, number of callus, number of embryogenic callus, and number of plantlet. However, transgenic line from OG#1 genotype was more responsive to cold pre-culture compared to the OS#9 genotype in all paramaters observed. The results of present study suggest that the anther of transgenic line OG#1 genotype had a greater potential to be used in producing homozygous plants and to develope doubling haploid. The potential of the anther is not controlled by the transformed gene in rice plants, but rather the host plant genotypes.

Keywords: Anther culture; Cold pre-culture; Homozygous; Haploid; Transgenic rice

Introduction

Anther culture is a useful technique in plant breeding to rapidly obtain the homozygous offspring and improve selection efficiency. Regeneration of haploid plants from anther culture provides new insight into conventional inbred method to develop pure lines in relatively a short period. However, the effectiveness of this technique depends on the efficiency of haploid plant regeneration from microspores within the anthers (Silva 2010). The basic principle of developing anther culture is starting from the inability of microspore cells to develop, which usually become gamete cells converted directly into complete plants (Nitsch 1981) in another sense, callus induction is influenced by the ability of anther to convert gametophyte pathways to sporophyte pathway.

Many studies has addressed to the success of anther culture in rice plants, especially related to its application in Japonica subspecies (Ozawa *et al.* 2003; Otani *et al.* 2005). However, it is mostly difficult to apply for Indica rice which has a recalcitrant genetic background and associate with low callus formation ability, limited morphogenesis potential or low callus regeneration ability and high percentage of albino formation (Bishnoi *et al.* 2000; Talebi *et al.* 2007). Knowing important aspects that can be modified in anther culture may lead to improve the genetic characteristics of Indica rice (Kumar and Murthy 2004; Dewi and Purwoko 2016a). Rapid development of anther culture is applied into biotechnology approaches such as gene transformation. This technique shorten the time required to obtain homozygous lines from transgenic plants (Datta *et al.* 2014).

Previously, we successfully developed transgenic rice overexpressing *OsWee1* gene in the indica subspecies as a host plant (Prasetyo *et al.* 2018). Screening of plants to obtain the second generation has been carried out. In order to shorten the screening time, and to get faster homozygous generation, the anther culture chosen will be followed by double-haploid to produce diploid plants. According to Dewi and Purwoko (2016b), this method can be used to produce pure lines in the first generation in less than a year.

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However, the success of anther culture is relatively low because the ability of anther to form callus and its regeneration into normal plants is difficult. The factors that influence the development of rice anther are genotype, age of donor plants and pre-culture treatments (Mishra and Rao 2016). Treatment by heat, cold (Khatun *et al.* 2012), physical, chemical and its adjusments to pre- and post-culture conditions can highly induce the development of anther culture (Silva 2010; Purnamaningsih and Ashrina 2011; Nurhasanah *et al.* 2016). The positive effect of the cold pretreatment for callus induction in anther is to delay the aging process of anther cell wall, increase cell divisions of pollen and induce important substances for androgenesis, mainly amino acids (Trejo-Tapia *et al.* 2002).

Previous studies have shown that the ability of anther regeneration from japonica rice subspecies is higher than indica subspecies. Mishra and Rao (2016) reported that only one out of 35 indica subspecies rice varieties were able to induce callus formation in the right medium for callus. This present research was conducted to determine the efficiency of cold pre-culture on the anther of two transgenic rice genotypes, and to find the potential of indica rice genotypes that can be used to produce homozygous line from anther culture in transgenic rice plants.

Materials and Methods

Plant materials and treatments

The plant materials that used in this study was two genotypes of transgenic rice T2 generation overexpressing of *OsWee1* gene obtained from previous study (Prasetyo *et al.* 2018), Genotipe OS#9 (Sertani backbone) and OG#1 (Gorontalo backbone) were developed at Central Laboratory for Biosciences, Polytechnic of Jember. Both genotypes belong to *Indica* subspecies. Plant material was prepared from the panicles, by harvesting the panicles which still covered by leaves in the morning at 8–10 am. Panicles were then wrapped in plastic wrapper and placed in the refrigerator at 4, 7 and 15ºC for cold pretreatment, and incubated for 7 days.

Anther planting

The middle parts of the panicles were used for anther culturing. The spikelets were pulled from panicle and surface sterilized using 96% alkohol for 30 sec and rinsed three times with sterilized water. Sterile spikelets were cut in one-fourth at the base while holding from the tip, then tapping it on the edge of the petridish until the anther were spread evenly on the surface of the culture media. The panicles husk must not fall on the surface of the media to minimize contamination.

Callus induction and regeneration

Anther were cultured on the N6 media (Nitsch 1981) supplemented with NAA (2 mg L^{-1}), Kinetin (2 mg L^{-1}), L-Proline (300 mg L^{-1}) and 3% of maltose placed in the petridish. Each petridish contains approximately 600 anthers which prepared from 100 spikelets. Cultures were placed in the dark condition at 25°C for callus induction, and examined every day during 30 days. After 8 weeks, the callus formed was counted and the embryogenic callus was transferred to regeneration media consisting of Murashig-Skoog (MS), Kinetin (2 mg L^{-1}), L-Proline (3 mg L^{-1}), Casein hydrolysate (500 mg L^{-1}), and 3% of maltose. Cultures were then incubated in growth chamber under a 16-h light/8-h dark photoperiod at 28°C.

Observations for callus induction and regeneration were carried out during 4 to 12 weeks after platting. The observation included percentage of anthers browning, estimated as follows: browning anther (%) = (number of anther browning/total number of anther plated) x 100%. The rate of callus formation was observed and recorded at 14 days after plating (DAP). The total number of callus formed, total number of embryogenic callus, and total number of plantlet were recorded for analysis. All data was subjected to analysis of variance (ANOVA) and significance means were determined using Duncan's Multiple Range Test (DMRT).

Genomic DNA extraction and PCR analysis

The rice DNA was isolated from 0.25 g of fresh leaves using an extraction buffer containing 0.35 *M* sorbitol, 0.1 *M* TRIS-HCl (pH 7.5), and 5 m*M* EDTA (pH 8), and crushed with liquid nitrogen. Isolation buffer containing 750 μL of $Na_2S_2O_5$ (3.8 g L^{-1}) was added to the scour and incubated for 1 hour at 65°C in water bath. During the incubation process, the tubes were slowly turned over every 15 min to complete the incubation process. The DNA was then purified by adding 750 μL of chloroform/isoamylalcohol (24:1). The suspensions were centrifuged at 10.000 rpm for 10 min and the supernatant were transferred into a new micro tube.

PCR analysis was performed with a total reaction of 20 μL containing 25 ng genomic DNA, 0.1 *M* dNTP mix, 1 unit of the Taq DNA polymerase enzyme (FastStart, Roche), RNase (0.1 μg μL^{-1}), and specific primers of *OsWee1* and *nptII* (0.25 μM). The genomic DNA was checking using nucleotides primers of *OsWee1*F-ATGGCACTTGGAATTAGTTGTGGTC and *OsWee1*R-TTATCGTGGCAAACCAACTGAGG. The primer nucleotide sequences of *nptII* were F-GTCATCTCACCTTGCTCCTGCC and R-GTCGCTTGGTCGGTCATTTCG. The amplification reaction was carried out using a PCR program consisting of pre-denaturation stages at 94°C for 2 min, followed by 25 cycles of denaturation at 94°C for 15 s, annealing at 57°C for 20 s and extension 72°C for 2 min. In the final stage the PCR process was carried out at the final extension at 72°C for 7 min. PCR products were applied by electrophoresis on 1% agarose gel. A total of 10 μL of PCR products and 1 μL of loading dye, and separated with a current of 100 Volts for

30 min. Furthermore, DNA was stained (soaked) by immersing the gel in a solution of ethidium bromide (1 mg L^{-1}) for 20 min and the DNA fragments resulting from the amplification were visualized using the Chemi-doc transluminator (Biometra).

Results

Callus development of anther culture

Percentage of anther browning: Browning is the appearance of brown in explants that inhibits growth and development. The anther browning percentage is used to test the anther viability with time limit of observation is 4 weeks after plantting (WAP). In general, anther which experiences browning after 4 WAP, the viability to produce embryogenic calli will be greatly reduced. Analysis of the percentage of anther browning at 4 WAP revealed that the cold pre-cultured had a very significant effect on the percentage of anther browning of the two transgenic genotypes. In both transgenic genotypes, cold pre-cultured at 4°C resulted in a lower percentage of anther browning compared to the treatments at 7°C and 15°C (Table 1). This suggest that there is a positive correlation between the cold pre-culture and the rate of anther browning. The lower temperature of cold pre-culture treatment was remarkably inhibited the rate of anther browning and vice versa. Anther of OS#9 genotype has a slower browning response than OG#1 genotype at all temperature treatments (Fig. 1).

The rate of callus formation: Callus is an amorphous cells resulting from explant poliferation. The anther forming callus is used to test the rapid of callus formation due to the treatment that has been given, and the observation keeps every week for 4 WAP. Anther of OG#1 genotype showed faster to form of callus formation compared to OS#9 genotype. Pre-culture treatment on OG#1 showed no significant difference in the rate of callus formation. The most effective cold pre-culture treatment on OS#9 and OG#1 genotypes was at 7°C which was able to induce callus formation in 25.87 and 18.81 days, respectively (Table 1). The lowest temperature treatment at 4°C, was not able to faster trigger the callus formation in both genotypes.

Total number of callus and embryogenic callus: This callus parameter refers to the number of callus formed at 10 WAP, which identified by callus formed from anther with a minimum size of ±2 mm. As shown in Table 1, cold pre-culture had a very significant effect on the number of callus formed at 10 WAP. The 7°C pre-culture temperature treatment at OG#1 produced the highest number of callus with an average of 67.12. The pre-culture temperature of 4°C on OS#9 produced the lowest number of callus, which was 2.44. Both genotype showed the optimum temperature to induce the appearance of the highest callus number was at 7°C, whereas at 4°C the growth of callus was suppressed in the both genotypes.

The embryogenic callus percentage parameter refers to the number of embryogenic callus formed from the anther at the age of 10 WAP. The embryogenic callus number was positive correlated with the number of calluses formed. In both genotype, cold pre-culture at 7°C highly induced the production of embryogenic callus. However, OG#1 was more responsive to cold treatment compared to the OS#9, by forming the highest number of embryogenic callus up to 65.87 (Table 1).

Regeneration of transgenic rice and analysis of gene expression

Technically plant regeneration is done by transferring the callus at the callus induction stage into plant regeneration media (Fig. 2). The parameter of total number of plantlet was determined based on the number of plantlets that formed at 12 WAP, with the characteristic that the plantlet arised from embryogenic callus which was able to form shoots either with roots or not. The treatment of cold pre-cultured on the number of plantlets showed that both genotypes were able to produce the highest number of plantlets at 7°C compared to that 4°C and 15°C (Table 1). Pre-culture at 7°C was able to stimulate the high shoot formation in both genotypes, OS#9 and OG#1 by 6.63 and 59.43 shoots, respectively. However, the highest shoot formation was produced by OG#1. Cold pre-cultured at 4°C decreased the number of plantlet produced in both genotypes.

PCR analysis was performed using *OsWee1* and *nptII* primers. *NptII* is a marker gene for kanamycin resistance in transgenic plants. The appearance of two DNA bands in one lane of agarose gel indicates that the plant is positive transgenic. From the two haploid plant lines OG#1 and OS#9 that were screened, several positive transgenic events were obtained which contained DNA bands of *OsWee1* and *nptII* with the sizes of 1239 bp and 550 bp, respectively (Fig. 3).

Discussion

The anther culture technique in the rice plant breeding is aimed to accelerate the acquisition of pure lines, and to obtain superior plant in a shorter time. In this study, the anther culture technique procedure was carried out *in vitro* through two stages. First, is the induction stage of callus from the pollen of transgenic plants overexpression of *OsWee1* gene, and the second is regeneration stage of plants from callus to haploid plants.

The successful stage of callus induction from pollen is characterized by decreasing in the percentage of browning in pollen cultures. Browning is the change of explant colour due to quinone produced from metabolic processes that catalyzed by phenol oxidase enzyme. Increase in temperature exceeding the optimum limit will accelerate the metabolic activity. However, the subsequent increase in temperature beyond the working limit of enzyme will

Table 1: Influence of cold pre-culture on the induction of callus developments and regeneration from two transgenic rice genotypes. The percentage of anther browning and callus formation were recorded at 4 weeks after platting. Total number of callus and embryogenic callus were calculated 10 weeks after platting. The number of plantlet was recorded 12 weeks after cultured

Rice Genotypes	Cold Pre-cultured (°C)	Percentage of Anther Browning (%)	Rate of Callus Formation (DAP)	Number of Callus	Number of Embryogenic Callus	Number of Plantlet
	4	10.00 ± 0.18a	31.00 ± 0.35c	2.43 ± 2.62a	2.34 ± 2.52a	0.62 ± 0.67a
OS#9	7	18.40 ± 0.32ab	25.87 ± 1.78b	7.63 ± 1.12ab	7.31 ± 1.10ab	6.63 ± 1.31ab
	15	18.03 ± 0.37ab	28.19 ± 3.67bc	3.25 ± 3.50a	3.16 ± 3.42a	3.52 ± 3.78a
	4	22.50 ± 0.74bc	21.03 ± 2.69a	24.75 ± 6.08bc	24.47 ± 5.82b	23.42 ± 2.41bc
OG#1	7	34.16 ± 0.46bc	18.81 ± 0.81a	67.13 ± 9.17d	65.87 ± 9.06d	59.43 ± 5.12d
	15	38.72 ± 0.71c	19.72 ± 2.67a	40.87 ± 14.88c	39.75 ± 13.98c	36.90 ± 11.80c

Values followed by different letters in a column indicate significantly different at $P \leq 0.05$
Means ± SE, $n = 4$. DAP; day after platting

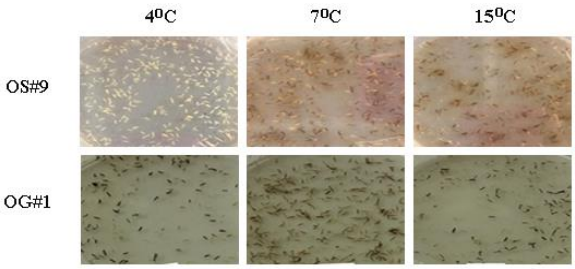

Fig. 1: Anther culture and anther browning percentage of transgenic rice OS#9 and OG#1 genotypes treated by cold pre-culture at 4°C, 7°C and 15°C for 14 hours. The pictures were taken 4 weeks after platting

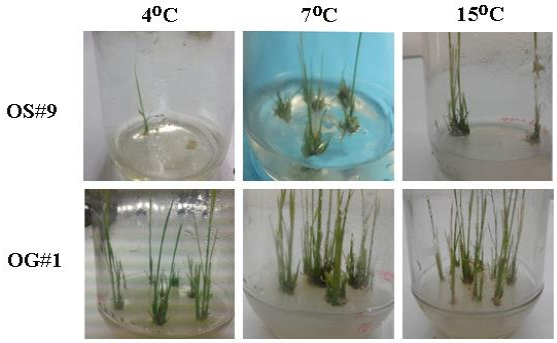

Fig. 2: Plant regeneration from transgenic rice OS#9 and OG#1 anthers in the variation of pre-culture temperature (4°C, 7°C and 15°C). The pictures were taken at 12 weeks after platting

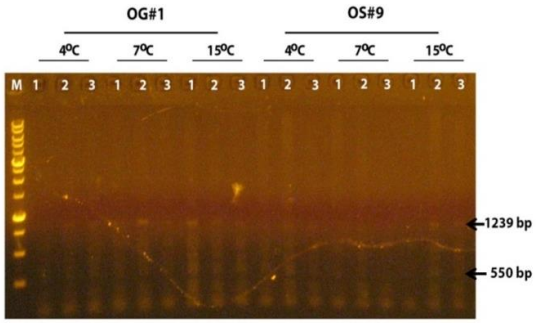

Fig. 3: Screening of transgenic T2 generation from haploid rice plants overexpressed *OsWee1* gene. Genomic DNA of OG#1 and OS#9 plants were isolated from cold precultured (4°C, 7°C and 15°C) with each three replicates (lane no. 1, 2 and 3). PCR analysis was performed using specific primers of *OsWee1* (1239 bp) and *nptII* (550 bp) to confirm the positive clones

caused decreasing of metabolic activities due to enzymes undergoing denaturation (Xa and Lang 2011). Pretreatment in rice panicles before inoculation was reported to be able to change the frequency of callus induction, and prevent anther from rapid browning due to the aging process of anther walls (Germana 2011). Other studies have reported that

low-temperature treatments can increase the frequency of callus formation, slow aging response, stimulate the formation of pre-embryos and harmonize cell conditions, as well as provides a time of anther wall tissue for microspore development (Ozawa *et al.* 2003). The results showed that response of OS#9 anther to turn browning was slower than OG#1 anther at the same temperature. This indicates that the browning response is not controlled by the *OsWee1* gene in the anther, but rather the basic genotype of rice used as a host for transformation. Lazaridou *et al.* (2016) reported that there was a very strong interaction between the genotype and the cold pre-treatment in several genomes of wheat anther. Each genome has a different response to temperature of pre-treatment. The difference was suspected because each genome has different genetic composition, the diversity of endogenous growth regulator and different biochemical processes that affect the response to temperature of pre-treatment. Silva and Ratnayake (2009) reported that anther culture response to the treatment is not only influenced by species differences in the genus, but also by differences in varieties within species.

The role of pre-culture in low temperature on the tissue metabolism is decreased metabolic rate due to reducing activity of several enzymes. Placement of tissues under normal *in vitro* condition would stimulate tissue to start a new metabolism pathway. Pre-culture by low temperature is important to change the gametophyte pathway to the sporophyte pathway of the microspore. The success of microspore culture is largely determined by the transition of microspores from the pathway of pollen formation to the path of embryo formation through pre-culture before cultured (Suaib and Arma 2012). Temperature shock is considered the most effective treatment for inducing the development of pollen embryogenesis. However, the optimum temperature and the duration of pre-cold treatment on each explant was not always the same and its influenced by plant genotypes. Dunwell (2010) stated that plant genotype is one of the endogenous factors that influence the success of anther culture. Other endogenous factor that has a significant influence on the induction of anther callus is the development stages of microspore (Germana 2011). The microspore in the late-uninucleate stage are reported greater induce callus rate formation and haploids (Li *et al.* 2016), because at this stage the pollen granulocyte is able to rapid differentiate into spores and it has stored abundant of nutrients (Wang *et al.* 2018). Zapata-Arias (2003) states that low temperature shock for up to 11 days can increase the frequency of callus formation up to 32% and it is useful for delaying the senescence. This statement is supported by Prayantini *et al.* (2013), that treatment of cold temperatures at 8-10°C for 8 days is reported to provide the best response to the formation of rice callus. Similar result was obtained in this study, that the rate of callus formation was induced faster at 7°C than the other temperature treatments in both genotypes of transgenic rice OS#9 and OG#1 by 25.87 and 18.81 days, respectively.

The efficiency of anther culture is determined by the producing of greenery tissues, as well as the double haploid plants that are produced both spontaneously and inductively. Production of green plants through anther culture is determined by the responsiveness to *in vitro* culture, successful callus induction, and regeneration of callus into green plants. Therefore, the ability to induce callus from microspores is a major factor determining the efficiency of anther culture (Zhao *et al.* 1996). The ability of each genotype to produce callus and regenerate into plants is different (Herawati *et al.* 2016), which was demonstrated by two genotypes tested in this study. The anther response in producing the highest callus was obtained from the OG#1 genotype at the 7°C pre-cultured. The same pattern was also obtained for anther ability to produce the embryogenic callus. The efficiency of anther culture associated with the production of green plants is expressed in the ratio of green plants to the number of callus capable in producing plants. The efficiency is also determined by the percentage of green plants to the total number of anthers being cultured.

Conclusion

In this study, cold pre-culture treatment was able to reduce the percentage of albino plants formation to zero percent. The ratio of total green plants number to the number of calluses formed at the optimum temperature of pre-culture (7°C) was 86.9% in OS#9 genotype, and 88.5% in OG#1 genotype. While the ratio of the number of green plantlets formed to the total anther inoculated in the two genotypes of OS#9 and OG#1 were 1.1% and 9.9%, respectively. Rukmini *et al.* (2013) reported that the cold pre-treatment on callus induction and plant regeneration in anther culture of rice was significantly affected by the genotype and the length period of pre-treatments.

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Author Contributions

NE and BS carried out the experiments and wrote the manuscript. MB designed the experiment and processing data. All authors approved the final manuscript.

Conflict of Interest

The all authors declare that they have no conflict of interest

Data Availability

All data reports in this work are available upon requesting to the corresponding author

Ethics Approval

Not applicable in this paper

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