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Doubled Haploid Rice Lines Production through Anther Culture of F_1 Derived from Abiotic Stress Tolerant Parents

(Pengeluaran Titis Beras Haploid Berganda melalui Kultur Anter Terbitan daripada Induk Tahan Tekanan Abiotik)

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ABSTRACT

Anther culture is one of the feasible technologies for accelerating rice breeding programs. This research aimed at determining the anther culture ability and doubled-haploid plants production of eight F_1s derived from single crosses between *indica* rice parents that had a single or multiple abiotic stresses tolerance. The experiment was conducted using completely randomized design (CRD) with 15 replications. Medium for callus induction was based on N6 + 2.0 mg L^{-1} NAA +0.5 mg L^{-1} kinetin + 1 mM putrescine, while the regeneration medium was based on MS + 0.5 mg L^{-1} NAA + 2.0 mg L^{-} kinetin ^{1} + 1 mM putrescine. Medium for rooting was MS + 0.5 mg L^{-1} IBA. The results indicated that all F_1 populations gave varied responses to callus induction and plant regeneration. The F_1 populations from HS4-11-1-2/B13926E-KA-23 had higher anther culture ability compared to other seven F_1 populations as indicated by the efficiency in callus formation and plant regeneration. Overall, the number of doubled haploid individuals (DHs) obtained was 158. F_1 populations using HS4-11-1-2 as female parent, HS4-11-1-2/B13926E-KA-23 and HS4-11-1-2/CG8-93-1-1 contributed 45.4% and 34.9% doubled haploid individuals, respectively. The developed DHs lines need to be morphologically and agronomically characterized as well as screened under different combinations of abiotic stress to identify high-yielding and abiotic stress tolerant individuals for further rice breeding program.

Keywords: Anther culture ability; callus induction; characterization; doubled haploid; rice breeding

ABSTRAK

Kultur anter adalah salah satu teknologi yang boleh dilaksanakan untuk mempercepatkan program pembiakbakaan padi. Kajian ini bertujuan untuk menentukan kemampuan kultur anter dan pengeluaran tanaman haploid berganda bagi lapan populasi F_1 yang berasal daripada persilangan tunggal induk padi *indica* yang tahan terhadap tekanan abiotik tunggal atau berganda. Uji kaji ini dijalankan menggunakan reka bentuk rawak lengkap (CRD) dengan 15 replikasi. Medium untuk aruhan kalus adalah berdasarkan N6 + 2.0 mg L^{-1} NAA+ 0.5 mg L^{-1} kinetin+ 1 mM putrescine, manakala medium penjanaan semula adalah berasaskan MS + 0.5 mg L^{-1} NAA + 2.0 mgL^{-1} kinetin + 1 mM putrescine. Medium untuk pengakaran ialah MS + 0.5 mg L^{-1} IBA. Keputusan menunjukkan bahawa semua populasi F_1 memberikan tindak balas yang berbeza-beza terhadap aruhan kalus dan penjanaan semula tumbuhan. Populasi F_1 daripada HS4-11-1-2/B13926E-KA-23 mempunyai keupayaan kultur anter yang lebih tinggi berbanding tujuh populasi F_1 lain seperti yang ditunjukkan oleh kecekapan dalam pembentukan kalus dan penjanaan semula tumbuhan. Secara keseluruhan, bilangan individu haploid berganda (DH) yang diperoleh adalah sebanyak 158. Populasi F_1 yang menggunakan HS4-11-1-2 sebagai induk betina, HS4-11-1-2/B13926E-KA-23 dan HS4-11-1-2/CG8-93-1-1 masing-masing menyumbang 45.4% dan 34.9% individu DH. Titisan DH yang dibangunkan perlu dicirikan secara morfologi dan agronomi serta disaring di bawah gabungan tekanan abiotik yang berbeza untuk mengenal pasti individu berhasil tinggi dan tahan terhadap tekanan abiotik untuk program pembiakbakaan padi selanjutnya.

Kata kunci: Aruhan kalus; haploid berganda; kemampuan kultur anter; pembiakbakaan padi

Introduction

Rice is one of the staple foods in Asia, including Indonesia. The population growth of approximately 1.25% per year (BPS 2020) has caused the high demand for rice. However, the attempt to increase rice production has become difficult on optimal land due to the increase of agricultural land conversion for other uses (Ilham, Syaukat & Friyatno 2005). In addition, because of the frequent occurrences of extreme weather worldwide, the conflicts between agricultural production and environmental resources are becoming increasingly intense which has led to the expansion of agricultural lands experiencing various abiotic stresses such as drought, submergence and salinity (Takeda & Matsuoka 2008; Yorobe et al. 2016). The combination of multiple abiotic tolerance traits in one rice variety is needed to overcome those problems that may occur in one growing season. Currently, rice varieties that have multiple abiotic stress tolerance are not yet available in Indonesia. Therefore, it is necessary to breed lowland rice which has high yields and is multi-tolerant to abiotic stress. The multi-tolerance to these abiotic stresses is one of the characters included in the Green Super Rice breeding program (Ali et al. 2017).

In Indonesia, rice varieties that are tolerant of salinity are Dendang, Lambur, Siak Raya, Inpari 34 Salin Agritan and Inpari 35 Salin Agritan, while varieties that are tolerant to drought are Batutegi, Inpari 38, Inpari 39, Inpari 41 and Inpago. Meanwhile, the submergence tolerant varieties are Inpari 29 and Inpari 30 Ciherang *Sub*1 (BBPADI 2018). Generally, the lowland rice varieties that have been released by the Indonesian Center for Rice Research have a single abiotic stress tolerance, while the varieties tolerant to dual or multi stresses are not yet available.

The development of new rice varieties usually takes at least 10 years when using conventional breeding. One time-consuming component is the line fixation stage in selection process to obtain pure lines. This is due to the heterozygous breeding materials. On the other hand, haploidization is another widely-used method in plant breeding due to its advantages to obtain that pure lines in the shortest possible time from heterozygous genetic materials such as F_1 population (Collard et al. 2017; Lenaerts et al. 2019). An alternative method of haploidization to obtain pure lines rapidly is through anther culture technique. By applying anther culture to F_1 population, homozygous lines in the form of spontaneous doubled haploid (DH) plants can be obtained in an early generation (Dewi & Purwoko 2012; Niazian & Shariatpanahi 2020). Therefore, it is apparent that the use of anther culture may also minimize costs while reducing time and labor.

Factors that influence the success of anther culture include plant genotype, media composition, pre-treatment of anther before culture, the development stage of microspore within the anther, the physiological condition of the anther donor plants and the time of young panicle harvesting (Tripathy et al. 2019). The plant genotypes that are commonly used in rice breeding programs in Indonesia are *indica* subspecies which have recalcitrant characteristics when cultured *in-vitro*, so it may be difficult to obtain green plants through anther culture (Dewi et al. 2004). Previous research found that the recalcitrance of *indica* subspecies was due to lower callusing and regenerating abilities when compared to *japonica* subspecies, particularly under different *in vitro* tissue culture conditions. Mostafiz and Wagiran (2019) reported that within the *indica* subspecies, genotypes dependency occurred in *in vitro* culture response.

However, in Indonesia, anther culture technique assisted by rapid testing for abiotic stresses has been widely used to obtain rice lines with various single tolerance traits such as shade tolerant (Mara et al. 2015; Sasmita et al. 2002), aluminum tolerant (Dewi, Purwoko & Bakhtiar 2006), drought tolerant (Akbar et al. 2018; Dewi et al. 2017; Gunarsih et al. 2016) and salinity tolerant lines (Anshori et al. 2021, 2019, 2018).

In order to accelerate the development of high yielding rice varieties having multiple tolerance to abiotic stresses, a conventional breeding assisted by anther culture technique is conducted. This research aimed at determining the rice anther culture ability and spontaneous doubled-haploid individuals production of eight F1 populations derived from the crossing of two indica parents that had a single or multiple abiotic stresses tolerance.

Materials and Methods

Plant materials

The experiment was conducted at the Tissue Culture Laboratory and Greenhouse of the Indonesian Center for Agricultural Biotechnology and Genetic Resources Research and Development (ICABIOGRAD) in Bogor, Indonesia from August 2018 until July 2019. The materials used were anthers from eight F_1 populations derived from single cross between two indica subspecies that have single or multiple tolerance to abiotic stresses (Table 1). The parents used in the crosses are

elite lines, namely IR86384-46-3-1-B (submergence tolerance, salinity tolerance) (IRRI 2015); HS4-11-1-2 (submergence tolerance, salinity tolerance) (Anshori et al. 2018; Safitri et al. 2016); B13926E-KA-23 (drought tolerance, submergence tolerance) (Wening. 2020); DR5-83-1-3 (drought tolerance) (Gunarsih et al. 2016); CG8-93-1-1 (drought tolerance, high yield) (Akbar et al. 2018; Gunarsih et al. 2016); FL478 (salinity tolerance, good agronomic characters) (Sexcion et al. 2009); and released varieties, namely Inpari 30 *Sub1* (superior varieties, submergence tolerance) (Septiningsih et al. 2014); and IR64 Dro1 variety (superior variety, drought-tolerance) (Uga et al. 2011).

TABLE 1. Genetic materials used as anther donor plants

F_1 Population	Cross Combination	Parental for F_1 population	
		Female	Male
CGH1	HS4-11-1-2/ B13926E-KA-23	➢ DH line derived from anther culture ➢ Tolerant to submergence and salinity	➢ Elite line derived from conventional breeding ➢ Tolerant to drought and submergence
CGH2	HS4-11-1-2/ CG8-93-1-1	➢ DH line derived from anther culture ➢ Tolerant to submergence and salinity	➢ DH line derived from anther culture ➢ Tolerant to drought and high yield
CGH3	DR5-83-1-3/ IR86384-46-3-1-B	➢ DH line derived from anther culture ➢ Tolerant to drought	➢ Elite line of conventional breeding ➢ Tolerant to submergence and salinity
CGH4	DR5-83-1-3/ B13926E-KA-23	➢ DH line derived from anther culture ➢ Tolerant to drought	➢ Elite line of conventional breeding ➢ Tolerant to drought and submergence
CGH5	FL478/ B13926E-KA-23	➢ Elite line of conventional breeding ➢ Tolerant to salinity, good agronomy	➢ Elite line of conventional breeding ➢ Tolerant to drought and submergence
CGH6	Inpari 30 Ciherang *Sub1*/ DR5-83-1-3	➢ The popular variety of Ciherang which has *Sub1* gene for tolerant to submergence ➢ High yield, good agronomy, tolerant to submergence	➢ DH line derived from anther culture ➢ Tolerant to drought
CGH7	IR64 *Dro1*/ IR86384-46-3-1-B	➢ The popular variety of IR64 which has deep-rooted *Dro1* gene for tolerant to drought ➢ Tolerant to drought	➢ Elite line derived from conventional breeding ➢ Tolerant to submergence and salinity
CGH8	IR64 *Dro1*/ HS4-11-1-2	➢ The popular variety of IR64 which has deep-rooted *Dro1* gene for tolerant to drought ➢ Tolerant to drought	➢ DH line derived from anther culture ➢ Tolerant to submergence and salinity

EXPERIMENTAL DESIGN AND MEDIA PREPARATION

The experiment was conducted using a completely randomized design (CRD) with 15 replications. The treatments used were eight F_1 populations (Table 1). Each experimental unit consisted of a petri dish containing anthers from 25 spikelets. The medium used for callus induction was N_6 medium supplemented with 2.0 mg L^{-1} NAA and 0.5 mg L^{-1} kinetin, while the callus regeneration medium was MS medium supplemented with 0.5 mg L^{-1} NAA and 2.0 mg L^{-1} kinetin. Putrescine 10^{-3} M was added to callus induction and regeneration media. Sucrose was added as much as 60 g L^{-1} and 40 g L^{-1} into the callus induction medium and the regeneration medium, respectively. Meanwhile, the rooting medium used was MS medium supplemented with 0.5 mg L^{-1} IBA and 30 g L^{-1}sucrose. Phytagel ™ 3 g L^{-1} used as solidifying agent and the pH of the media was maintained at 5.8. The media was sterilized in an autoclave for 20 minutes at a temperature of 120 °C and the pressure of 20 psi.

ANTHER CULTURE

The rice anther culture method followed Dewi et al. (2004). Young panicles were cold pretreated at 5 ± 2 °C and incubated for 8 days in the dark. Spikelets were removed from the sheath leaf after cold pretreatment and surface sterilized with 20% commercial bleach. Spikelets were cut at the base with scissors to allow the anther filaments to be cut. The panicle's central spikelets yielded anthers containing mid-to-late uninucleate microspores. Using a forcep, each spikelet was picked up by the uncut end, and the anthers were released on the callus induction medium by tapping the forcep on the Petri dish's rim. To induce callus formation from microspores, the cultures were kept in the dark at 25 ± 2 °C. To induce green plant regeneration, androgenic calli of 1-2 mm diameter were transferred onto MS regeneration media and incubated under light at 25 ± 2 °C.

PLANT ACCLIMATIZATION

Acclimatization was carried out by planting the green plantlets in a test tube filled with water for ± 1 week, then the plants were transferred to a seedling tray filled with muddy soil for 1 week. Plants were placed under light conditions with gradually increasing light intensity during the acclimatization process to harden them. After hardening, the rice seedlings were then transferred to the pots and grown in a greenhouse.

OBSERVATION AND DATA ANALYSIS

Observations were made on the onset of calli formation, the number of calli formed (CF), the number of calli producing plants (CPP), the number of calli producing green plants (CPG), the number of calli producing albino plants (CPA), the number of green plants (GP), the number of albino plants (AP), and the number of doubled-haploid plants (DH). The primary data obtained were used to obtain the percentage of anther producing callus (PAPC), callus induction efficiency (CIE) based on the number of calli to the number of anthers plated, total number of plants (TNP), percentage of green plants to the total number of plants (PGP), the percentage of albino plants to the total number of plants (PAP) and the green plant production efficiency of anther culture (GPPE) based on the percentage of green plants to the number of anthers plated. Data were analyzed using analysis of variance (ANOVA). If there were differences between treatments, it was analyzed further with Duncan's Multiple Range Test (DMRT). Analysis was carried out using SAS 9.0 version.

Results and Discussion

CALLUS INDUCTION

The callus induction and plant regeneration capacity are important in rice anther culture ability. The results showed that all characters differed significantly among the F_1 population (Table 2). All eight F_1 populations were able to produce callus. In general, the onset of callus formation ranged from 35.7 to 48.5 days after anther plating (DAP) (Table 3). CGH3 and CGH5 took the longest time in callus initiation (48.5 days) compared to the other six F_1 populations which took less than 40 days. The onset of callus formation is still in the range of previous *indica* rice anther culture reported by Dewi et al. (2017), Gunarsih et al. (2016), and Safitri et al. (2016). However, the onset of callus formation in this study takes longer time than that of rice anther culture of subspesies *japonica*, which usually takes 21 DAP (Ali et al. 2020).

The calli formed from this study varied. In the regeneration medium, there were two types of calli that could be identified by their structures and colours. Prior to the development of somatic embryos, embryogenic calli were creamy, compact, globular in shape, rapidly developing, and with clearly developed green spots, whereas non embryogenic calli were white, often friable or watery (Dewi, Putri & Purwoko 2019). The observation

TABLE 2. Means square of callus induction and plant regeneration from eight F_1 populations in rice anther culture

Sources of variation	df	OCF	CF	CPG	CPA	CPP	GP	AP	TNP
F_1 population	7	426.7**	5.3**	1.4**	0.5*	1.4**	9.6**	6.2**	7.8**
Error	112	87.9	0.8	0.1	0.2	0.2	0.7	2.0	1.5

*, ** indicate significant at $p \leq 0.05$ and $p \leq 0.01$, respectively; OCF: the onset of callus formation; CF: number of callus formed; CPG: number of callus producing green plants; CPA: number of callus producing albino plants; CPP: number of callus producing plants; GP: number of green plants; AP: number of albino plants; TNP: total number of plants

showed that some of the calli were white, also slightly transparent, while the other calli had a yellowish-white colour (Figure 1). According to previous studies, white colour calii regenerated albino plantlets, while calli with a creamy colour regenerated green plantlets (Dewi et al. 2004; Safitri et al. 2010). Only the creamy colour compact calli were able to pass through the regeneration stage into plants (Naik et al. 2016), while the watery callus

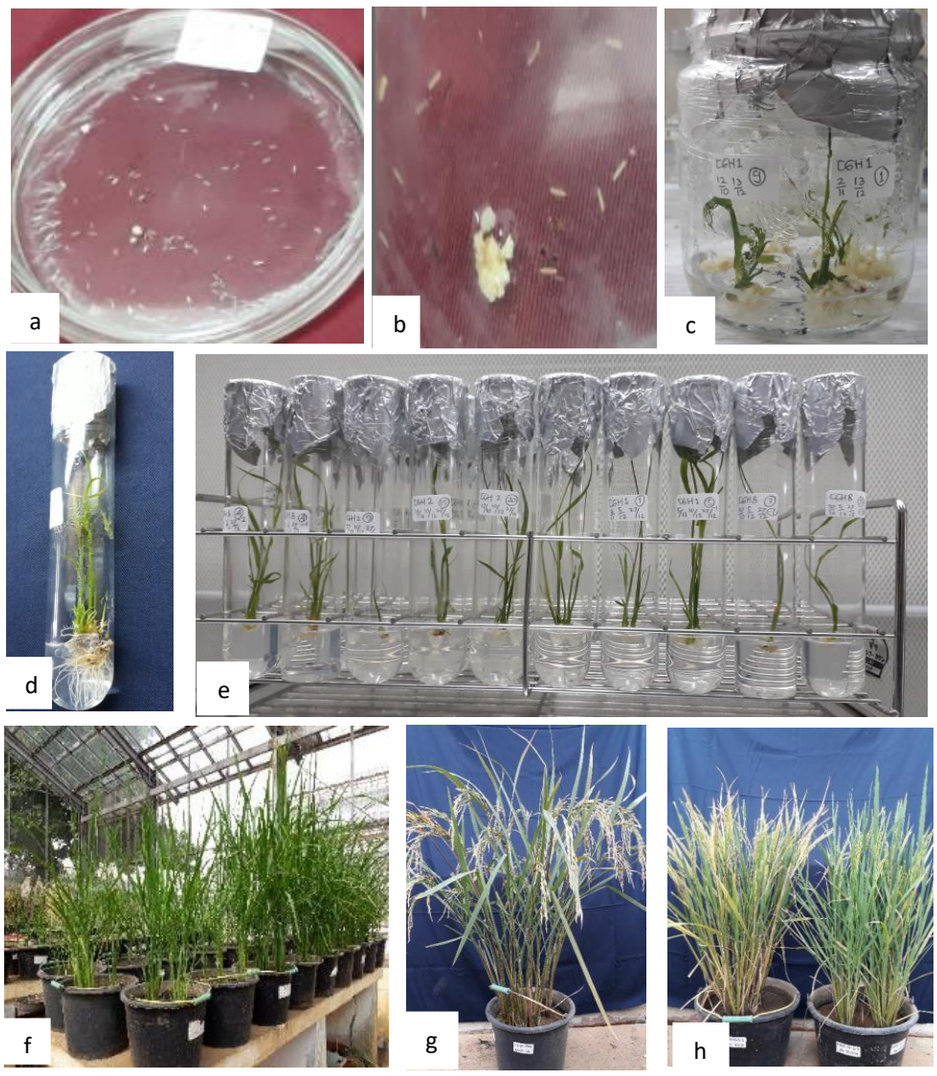

FIGURE 1. Rice anther culture; (a-b) Calli induced from microspores within the anther; (c) Green and albino plantlets on regeneration media; (d-e) Green plantlets on rooting media; (f) Hardening of green plant on the pots; (g) Doubled haploid plants and (h) Haploid plants selected after greenhouse grow out

was unable to regenerate. In another experiment, the calli size also important in obtaining green plantlets. Sasmita, Dewi and Purwoko (2001) reported that after transferring calli larger than 4 mm in diameter onto plant regeneration medium, they typically browned and perished, however when those calli eventually made it into plant regeneration medium, only albino plantlets were formed.

The eight F_1 populations tested in this study showed varying abilities in producing callus ranged from 3.3 to 14.6 calli per petri dish (Table 3). The highest number of calli is produced by CGH1 (14.6 calli), which is significantly different from CGH3, CGH4, CGH5, CGH6, CGH7 and CGH8, but not significantly different from CGH2 (11.9 calli per petri dish). The number of calli produced is the first important step when anther culture technique is adopted in rice breeding (Dewi, Safitri & Purwoko 2020). The increase in callus production will increase the chances of obtaining green plants. By obtaining a large number of green plants, the possibility of obtaining pure lines will be greater (Dewi & Purwoko 2008; Germana 2011).

Sasmita et al. (2002) reported that the selection of female parents with good anther culture ability was a factor that needs to be considered in determining F_1 parents in rice anther culture. Rice anther culture of hybrid from crossing between two desirable parents (F_1) generally showed better anther culture ability than anther culture of one parent alone (Safitri et al. 2010). However, the response of each genotype to anther culture technique varied, either within species, subspecies or varieties. Many researchers have reported the specificity of the genotype to callus induction especially in anther culture of *indica* rice subspecies (Talebi et al. 2007). *Indica* subspecies showed low callus growth, low regeneration ability and high percentage of albino plants, while *japonica* subspecies was more responsive to microspore embryogenesis than the *indica* type (Dewi & Purwoko. 2008; Grewal, Manito & Bartolome 2011; Samantaray et al. 2021). Moreover, Gueye and Ndir (2010) reported that *O. glaberrima* rice had more potential to produce callus and green plant regeneration than *O. sativa.*

TABLE 3. Mean of callus induction ability from eight F_1 populations in rice anther culture

F_1 population	The onset of callus formation (days)	Number of callus formed (CF)
CGH1	36.9b ± 3.7	14.6a ± 8.1
CGH2	39.3b ± 9.5	11.9ab ± 7.0
CGH3	48.5a ± 14.3	3.3e ± 1.8
CGH4	40.4b ± 8.3	6.8cde ± 4.8
CGH5	48.5a ± 12.5	4.9de ± 2.1
CGH6	35.7b ± 7.7	6.5cde ± 4.0
CGH7	36.0b ± 7.2	9.3bcd ± 6.7
CGH8	36.9b ± 7.6	10.1bc ± 7.7

The numbers in one column followed by the same letter are not significantly different in the DMRT test at the 5% level

PLANT REGENERATION

In anther culture, the microspores-derived calli will be transferred onto regeneration medium. Generally, not all calli will regenerate plants, some of them will become senescence and only a small amount will regenerate green and albino plants (Dewi, Putri & Purwoko 2019). In this research, more than 25% of the calli from each F_1 population regenerated into plants (Table 4). CGH1 had

the callus with the highest plant regeneration ability significance to the other F_1 populations, as many as 5.2 calli or 35.6% of the total callus. This population also had the highest number of calli produced green and albino plantlets. The calli that were able to produce green plants ranged from 8% to 34.9% and was relatively similar to those that produced albino plants which ranged from 7.8% to 30% (Table 4).

TABLE 4. Mean of the number of callus producing plant of eight F_1 populations for multi-tolerant abiotic stress

F_1 population	CPP	PCPP (%)	CPG	PCPG (%)	CPA	PCPA (%)
CGH1	5.2a±3.3	35.6±26.2	2.9a±1.2	19.6 ±24.0	2.3a±2.6	16.0±14.2
CGH2	3.1b±1.9	25.7±19.0	1.8bc±1.4	15.1±17.7	1.3b±1.4	10.6±12.3
CGH3	1.3c±0.5	38.0±33.3	0.3d±0.5	8.0±14.8	1.0b±0.5	30.0±33.9
CGH4	1.7bc±0.8	25.5±26.4	1.2c±0.4	17.6±25.6	0.5b±0.6	7.8±13.7
CGH5	1.9bc±1.1	38.4±16.1	1.1c±0.5	23.3±12.6	0.7b±1.0	15.1±15.6
CGH6	2.4bc±2.1	37.1±16.3	1.1c±1.3	16.5±21.1	1.3b±1.2	20.6±11.2
CGH7	2.9b±1.9	30.9±31.6	1.8bc±0.9	19.4±35.3	1.1b±1.4	11.5±9.7
CGH8	2.8b±1.4	40.1±29.6	2.1b±0.8	34.9±31.5	0.7b±1.1	6.6±10.3

The numbers in one column followed by the same letter are not significantly different in the DMRT test at the 5% level. CPP: number of callus producing plants; PCPP: percentage of callus producing plants; CPG: number of callus producing green plants; PCPG: % of callus producing green plants; CPA: number of callus producing albino plants; PCPA: % of callus producing albino plants

Plant regeneration in anther culture-derived calli is presented in Table 5. The total number of plants regenerated from the eight F_1 populations varied in a range of 6.5 - 24.8 plants. CGH1 produced the highest total number of plants that was significantly different from the other seven F_1 populations. In this research, like in any other rice anther culture research, the green plant regeneration in all populations is always coupled with albino plant regeneration. The number of green and albino plantlets regenerated varied significantly depending upon the genotypes of the anther donor plants. Based on the total number of plant, percentage of green plant regeneration ranged from 5.7 to 76.3%, while percentage of albino plant regeneration ranged from 23.7% to 94.3%. Anther culture of *indica* and *japonica* subspecies always produced a lower proportion of green plants than albino plants (chloroplast mutant) (Dewi & Purwoko 2008; Dewi et al. 2004). Dewi et al. (2019) observed that in the *indica* rice anther culture, a significant effect occurred on the percentage of callus producing green plants, but had no significant effect on the percentage of callus that produced albino plants. This is because the majority of the resulting individuals are albino compared to green plants. Therefore, albinism has remained a major challenge that hinders the application of anther culture and microspore culture technology in many breeding programs of Gramineae family, especially for the *indica* rice subspecies (Ali et al. 2020; Dewi & Purwoko 2012; Zhou 1996).

ANTHER CULTURE ABILITY

Callus induction and green plant production efficiency are two important features in incorporating the anther culture technique into the rice breeding program (Dewi & Purwoko 2012). The results of this study showed that generally the high efficiency of callus induction was in line with the high efficiency of green plant production (Table 6). The percentage of the number of callus to the number of anthers cultured from each F_1 population was expressed in terms of the efficiency of callus induction (CIE). CGH1 showed the highest callus induction efficiency (11.8%), followed by CGH8 and CGH2 of 8.2% and 8.1%, respectively. The other five

TABLE 5. Mean of the plant regeneration in rice anther culture of eight F_1 populations

F_1 population	GP	PGP (%)	AP	PAP (%)	TNP
CGH1	10.6a±4.2	42.7± 30.7	14.2a±17.6	57.3± 30.7	24.8a±18.3
CGH2	7.3ab±5.9	61.5±30.9	4.6b±5.1	38.6±30.9	11.9b±8.0
CGH3	0.5d±0.9	5.7±34.8	7.7ab±4.9	94.3±34.8	8.1b±4.3
CGH4	5.4bc±4.7	72.9±30.2	2.0b±2.5	27.0±30.2	7.4b±4.6
CGH5	3.3bcd±2.6	50.0±35.2	3.3b±5.1	50.0±35.2	6.5b±6.0
CGH6	2.4cd±3.9	24.7±36.3	7.3ab±7.5	75.3±36.3	9.7b±10.0
CGH7	6.7ab±5.3	47.2±37.7	7.5ab±11.8	52.8±37.7	14.3b±13.5
CGH8	9.9a±9.9	76.3±27.4	3.1b±6.0	23.7±27.4	12.9b±10.3

The numbers in one column followed by the same letter are not significantly different in the DMRT test at the 5% level.GP: number of green plants; PGP: percentage of green plants; AP: number of albino plants; PAP: percentage of albino plants; TNP: total number of plants

F_1 populations had CIE ranged from 2.7% to 7.1%. The percentage of green plants obtained to the number of anthers plated was expressed as the green plant production efficiency (GPPE). The highest GPPE belonged to CGH1 (8.6%), followed by CGH8 (7.9%), and CGH7 (5.1%). Meanwhile, the other F_1 populations had GPPE less than 5.0% (Table 6). The GPPE in this research was better (0.4 - 8.6%) when compared to the previous research reported by Safitri et al. (2016) which only reached 0 - 3.1%.

It is interesting to note that based on both callus induction and green plantlet production efficiency, CGH1 derived from HS4-11-1-2/B13926E-KA-23 had the highest anther culture ability. As reported previously by Safitri et al. (2016), HS4-11-1-2 was a doubled haploid line. This line was the result of anther culture of F_1 derived from a cross between IR77674 and INPARI 29 which also has the highest anther culture efficiency. According to Yamagishi et al. (1998), one region in chromosome 1 was found to control callus formation from microspore, and one region in chromosome 10 appeared to control the ratio of green to albino regenerated plants.

TABLE 6. Efficiency of callus induction and green plants production in anther culture

F_1 population	CIE (%)	GPPE (%)
CGH1	11.8	8.6
CGH2	8.1	4.9
CGH3	2.7	0.4
CGH4	5.6	4.4
CGH5	3.9	2.6
CGH6	5.4	2.0
CGH7	7.1	5.1
CGH8	8.2	7.9

Note: CIE = callus induction efficiency; GPPE = green plant production efficiency

DOUBLED HAPLOID PLANT PRODUCTION

The total number of green plants from anther culture of eight F_1s derived from crosses of abiotic stress tolerant parents that were successfully acclimatized in this research was 508 plants out of 902 green plants (Table 7). The average plant acclimatization success rate was 56.3%, with a range between 41.9% and 89.5%. CGH5 had the highest acclimatization success rate (89.5%) followed by CGH2 (63.7%). Plants produced from anther culture included haploid plants, doubled-haploid (DH) plants, polyploid and plants that have variations in ploidy levels or mixoploid (Dewi & Purwoko 2011; Zhang 1989). In this study, identification of spontaneous doubled-haploid plants was done by comparing all plants derived from anther culture to normal diploid rice plants through phenotypic marker, such as the differences in plant height, leaf shape, panicle sterility, and the existence of ligule and auricle. Spontaneous doubled-haploid rice plant similar to diploid rice plant have fertile panicle, ligule and auricle, but have shorter height and narrower leaf shape than the plants with higher ploidy level (Dewi & Purwoko 2012; Safitri et al. 2010).

In other experiment, Mishra et al. (2015) also determined the ploidy of regenerated plants based on visual observations of morphology and fertility. Haploid plants were marked by their small habitus, less vigourous, and most importantly sterile when compared to diploids, which indicated normal growth and fertility. Mayakaduwa and Silva (2019) compared the ploidy of the anther culture regenerants selected by phenotypic marker with flowcytometry and stomatal observation. They found that confirmation by flowcytometry indicated that selection using phenotypic marker could be used to separate haploid, diploid, other ploidy and also mixoploid regenerants. Stomatal measurements also confirmed that lower ploidy status is indicated by smaller cell size and higher cell density.

TABLE 7. Acclimatization of green plants and dihaploid plants produced from rice anther culture

F_1 population	GPA	GPS	DH	PGPS (%)	PDH (%)
CGH1	206	108	49	52.4	45.4
CGH2	135	86	30	63.7	34.9
CGH3	12	6	1	50.0	16.7
CGH4	102	49	16	48.0	32.7
CGH5	57	51	11	89.5	21.6
CGH6	43	18	5	41.9	27.8
CGH7	132	70	20	53.0	28.6
CGH8	215	120	26	55.8	21.7

GPA = number of green plants acclimatized; GPS = number of green plant survive after acclimatization; DH = number of doubled-haploid plants; PGPS = percentage of green plant survive after acclimatization; PDH = percentage of doubled-haploid plants

The DH plants obtained from this research (Table 7) were the result of spontaneous chromosome doubling as previously found in cereal, especially rice (Germana 2011; Seguí-Simarro & Nuez 2008a). The DH plants obtained through spontaneous chromosome doubling from this study were 158 plants or 31.1%. The highest DH plants production belonged to CGH1 (45.4%) followed by CGH2 (34.9%). Spontaneous duplication of chromosomes was of great importance as an alternative to artificial chromosome duplication, however the doubling rate varied greatly between genotypes (Kleiber et al. 2012). Chaikam et al. (2019) found that in maize, haploid fertility may be spontaneously restored by natural chromosomal doubling but at a rate too low for practical applications. However, the frequency of spontaneous chromosome doubling in rice can be found

to be over 50% (Naik et al. 2016; Seguí-Simarro & Nuez 2008b). The more DH plants obtained, the higher opportunities to get homozygous plants that have high yields, good agronomic performance and tolerance to abiotic stresses.

Conclusion

This research confirmed that callus induction and plant regeneration capacity in rice anther culture is genotype dependent. Based on callus induction and green plant regeneration efficiency, F_1 population that had the highest anther culture ability was CGH1. This might be due to the use of doubled haploid line as one of its parent. The number of green plants that were successfully acclimatized was 508 plants, while the number of spontaneous doubled-haploid plants obtained was 158 plants (31.1%). The highest number of spontaneous doubled haploid plants was obtained in CGH1 derived from HS4-11-1-2/B13926E-KA-23 (40 plants) followed by CGH2 derived from HS4-11-1-2/CG8-93-1-1 (49 plants). The production for high-yielding and abiotic stress tolerance rice is compulsory to address the issues of climate change and food security. Therefore, further screening and selection based on the high yielding and abiotic stress tolerance traits should be performed using the generated DH lines.

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