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Rice Anther Culture to Develop Double Haploid Population and Blast Resistant Lines

A. Dinar Ambarwati[1], Ida H. Somantri[1], Dwinita W. Utami[1], Aniversari Apriana[1], and Sugiono Moeljopawiro[2]

[1]Indonesian Center for Agricultural Biotechnology Research and Development, Jl. Tentara Pelajar 3A, Bogor 16111
[2]Indonesian Center of Plant Variety Protection, Jl. Harsono RM No. 3, Jakarta 12550

ABSTRAK

Kultur Anter Padi untuk Mendapatkan Galur-galur Tahan Penyakit Blas. ***A. Dinar Ambarwati, Ida H. Somantri, Dwinita W. Utami, Aniversari Apriana, dan Sugiono Moeljopawiro.*** Penyakit blas pada padi yang disebabkan oleh cendawan *Pyricularia grisea*, merupakan salah satu kendala dalam produksi beras. Sumber gen ketahanan terhadap penyakit blas dijumpai pada spesies padi liar *Oryza rufipogon*. Populasi silang ganda (BC_2F_3) turunan IR64 dan *O. rufipogon* mempunyai QTL untuk sifat ketahanan terhadap penyakit blas. Untuk mempercepat perolehan tanaman homosigot dari populasi tersebut, dilakukan kultur anter pada dua media induksi kalus: I_1 (N6 + NAA 2 mg/l + kinetin 0,5 mg/l + sukrosa 60 g/l + putresin 0,16 g/l) dan I_2 (N6 + 2,4-D 2 mg/l + sukrosa 50 g/l) dan dua media regenerasi: R_1 (MS + NAA 0,5 mg/l + kinetin 2 mg/l + sukrosa 40 g/l + putresin 0,16 g/l) dan R_2 (MS + NAA 1 mg/l + kinetin 2 mg/l + sukrosa 30 g/l). Kultur anter dilakukan pada sembilan genotipe, di mana tiga genotipe (149-16, 343, 337-13) memberikan respon terbaik dalam produksi planlet hijau setelah dikulturkan pada media regenerasi R_1. Dari 208 planlet hasil regenerasi diperoleh 42 planlet haploid ganda dari genotipe 149-16, 11 planlet haploid ganda dari genotipe 343, dan 44 planlet haploid ganda dari genotipe 337-13. Skrining ketahanan blas di rumah kaca pada populasi haploid ganda menghasilkan 46 tanaman tahan terhadap ras 001, 33 tanaman tahan terhadap ras 033, dan 79 tanaman tahan terhadap ras 173. Sebanyak 28 tanaman bersifat tahan, baik terhadap ras 001, 033, maupun 173 seperti halnya *O. rufipogon*. Galur-galur homosigot ini akan diuji di lapang untuk ketahanannya terhadap penyakit blas dan karakter agronominya.

Kata kunci: Kultur anter, IR64, *Oryza rufipogon*, ketahanan blas.

INTRODUCTION

Rice is grown under a wide range of agro-climatic conditions where several diseases, insect pests, and unfavorable weather affect its productivity. Rice blast disease caused by fungus *Pyricularia grisea* is a major constraint in rice production (Baker *et al*. 1997, Scardaci *et al*. 1997). Therefore, there is a continuous need to identify diverse sources of genes for tolerance to various stresses and develop improved rice germplasm with a wide genetic base. The wild relatives of cultivated rice (*Oryza sativa*) are an important reservoir of useful genes for resistance to major diseases and insects (Khush and Brar 1988, Vaughan 1994). *Oryza rufipogon*, one of the closest relatives of cultivated rice, has been found to contain genes that can substantially increase rice yields (Xiao *et al*. 1996). There are some notable examples of the successful transfer of alien genes into cultivated rice varieties including those for grassy stunt virus and brown plant hopper resistances, cytoplasmic male sterility, elongation ability, as well as blast and bacterial blight resistances (Khush and Brar 1991).

A previous study on the use of wild species *O. rufipogon* (IRGC #105491) and BC_2F_3 population progenies of IR64 x *O. rufipogon* cross indicated that quantitative inheritance of blast resistance under a greenhouse condition (Utami *et al*. 2005). To speed up finding new advanced rice lines that have potential yields equal to IR64 but with improved resistance to blast, we have developed homozygote rice lines using the anther culture technique.

The advantages of haploid culture, technique especially the anther culture, in the breeding program have been known (Khush and Virmani 1985, Zapata 1985, Raina 1989). Anther culture is an important technique to develop true breeding lines in the immediate generation, thereby shortening the breeding cycle of new varieties and allows early expression of recessive genes. The selection efficiency is considerably increased as the population contains only fixed homozygous lines. Beside the advantages of using the techniques, anther culture has a disadvantages and constraints, i.e., low efficiencies of callus production, low plant regeneration, and high proportion of albino plants produced.

Utilization of anther culture techniques in rice breeding programs has been in practice for more than 20 years. In China, more than 81 rice varieties and promising lines have been developed (Hu 1985). Disease resistant rice varieties "Hwangeongbyeo" were developed in Korea (Koh *et al*. 1993). Double haploid lines were produced to obtain fertile lines from interspecific crosses between *O. sativa* x *O. glaberrima* (Sarla and Swamy 2005). Interspecific hybrids of *O. sativa* x *O. rufipogon* and *O. sativa* x *O.*

longistaminata were used in anther culture to assess their potential in callus induction and regeneration (Rout and Sarma 1986). Some promising lines intermediate between the parents and possessing blast resistance have been obtained through anther culture (Li *et al*. 1983).

In the rice anther culture, N6 medium was formulated to raise the frequency of pollen callusing. The MS basal medium supplemented with different cytokinins was used in a series of regeneration experiments (Rout and Sarma 1986). Consistent plantlet regenerations from pollen calli in sufficient numbers enhances efficiency and use of the anther culture technique in high volume rice breeding.

This study was done to develop double haploid populations and screened the selected lines for their resistance to rice blast in a screenhouse.

MATERIALS AND METHODS

Development of Double Haploid Lines Through Anther Culture

Nine genotypes from three selected families BC_2F_3 of interspesific crossing between an *O. sativa* ssp. indica, IR64, as a recurrent parent and a wild rice species, *O. rufipogon* (IRGC 105491 from Malaysia) as a donor parent were used in the experiments. These genotype were numbered as 149-1, 149-8, 149-16, 149-22, 343, 337-6, 337-13, 337-17, and 337-20. All plants were grown in a screenhouse until the panicles were ready for collection. Panicles (boots) were collected and cold-treated at 8-10°C for 7-10 days. They were then surface sterilized with 20% (v/v) chlorox for 20 min. The spikelets were picked up with their open ends down using a forceps and were tapped on the rim of a Petri dish. Modified N6 media (Chu 1978) i.e: I_1 (N6 + 2 mg/l NAA + 0.5 mg/l kinetin + 60 g/l sucrose + 0.16 g/l putrecine) and I_2 (N6 + 2 mg/l 2,4-D + 50 g/l sucrose) (Hanarida *et al*. 2002a, 2002b, Dewi *et al*. 2004) were used as callus induction media.

About 20-30 days after transfering the plates into a dark condition, callus emerge from the dehisced anthers. Number of calli was calculated based on the plated anthers forming calli. Those that were 2 mm in size were transferred into regeneration media R_1 (MS + 0.5 mg/l NAA + 2 mg/l kinetin + 40 g/l sucrose + 0.16 g/l putrecine) and R_2 (MS + 1 mg/l NAA + 2 mg/l kinetin + 30 g/l sucrose) (Hanarida *et al*. 2002a, 2002b) and incubated under a continuous light at 25°C. Regeneration efficiency was calculated based on the plated anthers forming green plantlets. In order to strengthen the roots, the green plantlets were usually re-transferred to test tubes with rooting medium MS + 0.5 mg/l IBA + 30 g/l sucrose. The plantlets with vigorous roots were finally transplanted pots containing soil and fertilizer and grown to maturity in the screen house.

Anthers from 25 spikelets (about 125 anthers) were cultured in a 9 cm Petri dish containing the callus induction medium. There were unbalanced number of anther for each media treatment depending upon the availability of the anthers. The media response to calli and green plantlet production were analysed using one-sample T test for unbalanced data.

Screening for Blast Resistance in the Screenhouse

Seed from double haploid plants (the first generation/Ho) were harvested and planted as H1 generation in the screenhouse for blast resistance screening. A completely randomized design was used in the screening. Plants were inoculated with three Indonesian dominant races (001, 033, and 173) (Utami *et al*. 2000) at three to four leaf stages or about 18-21 days after planting. Three groups with the same population were prepared and inoculated with each race for each group. Inocula were prepared on oatmeal agar media and adjusted to a concentration of 3×10^5 spores per ml. Variety Kencana Bali, wich is susceptible to all P.o. races was used as a negative control, whereas wild rice *O. rufipogon* was use as a positive control. The experiments were repeated five times.

Inoculated plants were left in a moist chamber for about 48 hours to allow the attachment of spores to leaf surfaces. The plants were then placed in a humid screenhouse misted with water sprinkle's for about one week. After seven days of inoculation, the plants were taken out from the screenhouse and scored for blast disease intensities according to IRRI (1996), i.e., >1.6 = susceptible (S), $1.5\leq$ medium resistan ≥ 1 (MR), and <1 = resistan (R).

RESULTS AND DISCUSSION

The experiment a anther culture was conducted in 9 rice genotypes from three families of BC_2F_3 population, to assess the anther culture ability using two callus induction media: I_1 (N6 + 2 mg/l NAA + 0.5 mg/l kinetin + 60 g/l sucrose + 0.16 g/l putrecine) and I_2 (N6 + 2 mg/l 2,4-D + 50 g/l sucrose) and two regeneration medium: R_1 (MS + 0.5 mg/l NAA + 2 mg/l kinetin + 40 g/l sucrose + 0.16 g/l putrecine) and R_2 (MS + 1 mg/l NAA + 2 mg/l kinetin + 30 g/l sucrose). Seed of the genotypes were grown the first two or three tillers were collected from each plant with the outer leaves are discarded (Figure 1a). The spikelets

were picked up with their open ends down by a forceps and tapped on the rim of a Petri dish, causing anthers in the spikelets to vibrate and fall onto surface of callus induction medium (Figure 1b). After 20-30 days on medium, calli emerge from the dehisced anthers (Figure 1c). Those that were 2 mm in size were transferred a to the regeneration medium. Texture and colors of the calli reflect their green plantlet redifferentiation competences. Calli with a compact texture and light green color was more likely to generate green plantlets (Figure 1d), whereas white or milky calli usually differentiate albino plantlets (Figure 1e).

Result of the callus induction or regeneration indicated that all genotypes produced calli with percenttages varied from 0.02 to 2.88% (Table 1). Each genotype showed a different response to the anther culture. Only calli from three genotypes (149-16, 343, 337-13) could regenerate into green plantlets, although produced albino plants as well. The R_1 medium was significantly better at 5% confidence level (t^{test} = 2.227*) than R_2 in producing the green plantlets.

Based on results of the preliminary study, three most responsive genotypes (149-16, 343, 337-13) were selected for further anther culture experiment to maximize the production of green plantlet (Table 1). The response of callus formation and plant regeneration on the two media tested showed that medium containing NAA, kinetin, or 2.4-D with the addition of putrecine or without putrecine was effective in promoting callus formation (Table 2).

Vigorous growth of embryogenic calli were obtained from microcalli with 1-2 mm diameters after being transferred to the regeneration medium. In producing the green plantlets, the R_1 medium was significantly better at 5% confidence level (t = 2.821*) than the $R_{2,}$ and although albino plantlets still produced. The difference between the two medium was on the NAA, sucrose, and putrecine contents.

R_1 medium that contained 0.16 g/l putrecine was more effective in promoting callus regeneration than the R_2 medium as indicated by the highed number of green plantlet on genotype 337-13 followed by 149-16 and 343. Genotype and nutrient composition of the culture medium are the major source of variation under *in vitro* culture. There are several reports on modes of plant regeneration using different strategies, such as nutrient manipulation, partial desiccation of callus, manipulation of phytohormone levels, polyamines, carbohydrate source, and improved culture conditions (Bajaj and Rajam 1995, Khanna and Raina 1998).

Polyamines, spermidine, and spermine and their precursor putrecine were ubiquitous organic polycations involved in various plant growth and develop-

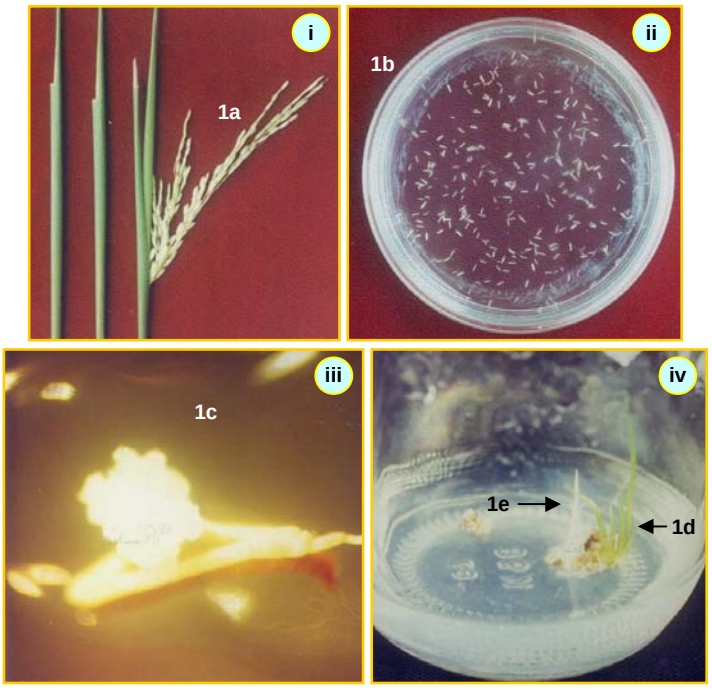

Figure 1. Steps on the production of plantlets from anther cultures. I = collecting panicles from the donor plant, ii = anthers cultured in a Petri dish containing the callus induction medium, iii = callus induction from the dehisced anthers, iv = calli that produce green and albino plantlets.

Table 1. Callus induction and plantlet regeneration on nine genotypes from three families BC_2F_3 of interspesific crossing between *O. rufipogon* and IR64.

Genotype	Callus induction media	No. of plated anthers	No. of anthers forming calli	Regeneration media	No. of green plantlet	No. of albino plantlet
149-1	I_1	447	6 (1.34)	R_1	-	-
	I_2	851	17 (2.00)	R_2	2 (0.24)	9 (1.06)
149-8	I_1	634	5 (0.79)	R_1	-	-
	I_2	1382	33 (2.39)	R_2	-	-
149-16	I_1	3969	73 (1.84)	R_1	22 (0.55)	22 (0.55)
	I_2	3888	48 (1.23)	R_2	1 (0.03)	8 (0.21)
149-22	I_1	2285	5 (0.22)	R_1	-	-
	I_2	2698	33 (1.22)	R_2	-	-
343	I_1	7156	117 (1.63)	R_1	16 (0.22)	123 (1.72)
	I_2	6704	193 (2.88)	R_2	12 (0.18)	109 (1.63)
337-6	I_1	3037	26 (0.86)	R_1	-	4 (0.13)
	I_2	3461	50 (1.44)	R_2	-	90 (2.60)
337-13	I_1	8512	140 (0.02)	R_1	52 (0.61)	26 (0.31)
	I_2	9714	208 (2.14)	R_2	32 (0.33)	213 (2.19)
337-17	I_1	1406	16 (1.14)	R_1	-	7 (0.50)
	I_2	903	8 (0.89)	R_2	-	4 (0.44)
337-20	I_1	413	7 (1.69)	R_1	-	-
	I_2	1106	8 (0.72)	R_2	-	7 (0.63)

I_1 = N6 + 2 mg/l NAA + 0.5 mg/l kinetin + 60 g/l sucrose + 0.16 g/l putrecine, I_2 = N6 + 2 mg/l 2,4-D + 50 g/l sucrose, R_1 = MS + 0.5 mg/l NAA + 2 mg/l kinetin + 40 g/l sucrose + 0.16 g/l putrecine, R_2 = MS + 1 mg/l NAA + 2 mg/l kinetin + 30 g/l sucrose.

Table 2. Calli inductions and plantlet regenerations on three selected rice genotypes of BC_2F_3 population.

Genotype	Callus induction media	No. of plated anthers	No. of anthers forming calli	Regeneration media	No. of green plantlet	No. of albino plantlet
149-16	I_1	13009	259 (1.99)	R_1	81 (0.62)	53 (0.41)
	I_2	9626	123 (1.28)	R_2	1 (0.01)	8 (0.08)
Total		22635	382 (1.69)		82 (0.36)	61 (0.27)
343	I_1	13495	230 (1.70)	R_1	23 (0.17)	177 (1.31)
	I_2	10229	293 (2.86)	R_2	12 (0.12)	109 (1.07)
Total		23724	523 (2.20)		35 (0.15)	286 (1.21)
337-13	I_1	10949	164 (1.50)	R_1	59 (0.54)	31 (0.28)
	I_2	13522	259 (1.92)	R_2	32 (0.24)	213 (1.58)
Total		24471	423 (1.73)		91 (0.37)	244 (1.00)

I_1 = N6 + 2 mg/l NAA + 0.5 mg/l kinetin + 60 g/l sucrose + 0.16 g/l putrecine, I_2 = N6 + 2 mg/l 2.4-D + 50 g/l sucrose, R_1 = MS + 0.5 mg/l NAA + 2 mg/l kinetin + 40 g/l sucrose + 0.16 g/l putrecine, R_2 = MS + 1 mg/l NAA + 2 mg/l kinetin + 30 g/l sucrose.

ment processes including in somatic embryogenesis. The mechanisms of action of polyamines on the development processes were still unclear (Walden *et al*. 1997). Favorable modification of cellular polyamine titers and their putrescine: spermidine ratio by the addition of exogenous polyamines (putrescine, spermidine) led to the induction of plant regeneration in poorly responding genotypes of indica rice (Shoeb *et al*. 2001). Dewi *et al.* (2001, 2004) reported that the addition of poliamine increased the production of green plantlets.

The highest number of green plantlet was obtained in genotype 337-13 (91) followed by 149-16 (82) and 343 (35). For plant regeneration, however the high number of calli producing albino plants was observed on the three genotypes. Albino phenomenon was one of the constrains in anther culture technique for breeding program. Aside from genotype dependence, high frequency of albino and haploid plants, obstructed widespread use of the anther culture technology. The frequency of albino plants increases with the rise in incubation temperature and varied according to genotype and physiological conditions of the anther donor plants (Chung 1992, Sun and Zhao 1992). The regeneration frequencies of albino plantlets were 18.8-71.4% of calli obtained from anther culture of wild species that had resistances to rice blast, resistance to bacterial leaf blight, high protein content, and cold tolerance (Wu *et al*. 1987). Up to now, increasing the portion of double haploid lines and reducing the albino plants has been a major concern in anther culture researches (Chu *et al*. 2000).

In these experiment, the regeneration frequency ranged from 10 to 35.97% upon cultured anther on R_1 medium. Callus induction ranged from 0 to 18.6% upon culture of 45,400 anthers from 75 F_1s derived from crosses between *O. sativa* and *O. glaberrima*. Anthers of only 41 F_1s showed on an average 1.35% callus induction, and plant regeneration ranged from 0 to 77% (Sarla and Swamy 2005). Anther culture of interspecific hybrids between *O. sativa* x *O. rufipogon* resulted 20.8% of regeneration frequency, based on the number of calli regenerated to number of calli plated (Rout and Sarma 1986).

Based on the phenotypic evaluation, from a total of 208 green plantlets, 42 Double Haploid (DH) plants were produced from genotype 149-16, 11 DH plants derived from genotype 343, and 44 DH plants from genotype 337-13. A totall of 97 DH plants were produced from the three selected genotypes of BC_2F_3 population (Table 3).

The homozygous diploid plants regenerated from natural doubling of pollen plants, usually showed phenoltypically the same as the donor variety, comprise 50-65% among the regenerated plants. The haploid plants were smaller and have more tillers than the donor variety. They produced flowers but no seed setting, and they have no ligule and auricle (Zhang 1992). Morphological differences between the haploid and the diploid plants were distinct *in vivo*, the haploid plant possessed about two times more tillers, 60-70% of plant height, panicle length, and leaf length of the diploid plant (Chung 1992).

All 97 DH plants (the first generation/Ho) were planted in a greenhouse for seed multiplications. Seeds from those plants were harvested and planted as H1 generations in a screenhouse for blast resistance screening. The DH population showed varied reactions from susceptible to resistant (Table 4 and Figure 2) based on the IRRI Standart Evaluation System (IRRI 1996). Among 282 plants tested, 46 plants, 33 plants, and 79 plants were resistant to race 001, 033, and 173, respectivelly. Twenty eight plants were resistant to all races (001, 033, 173) the some as the wild rice *O. rufipogon*, indicating that they had a horizontal resistance. The resistance genes come from the wild rice *Oryza rufipogon* that have been conserved in new rice families with improved blast resistance. The double

Table 3. Production of green plantlets and double haploid plants from three selected genotypes of BC_2F_3 population.

Genotype	No. of plated anthers	No. of anthers forming calli	No. of green plantlet	No. of DH plant
149-16	22635	382 (1.69)	82 (0.36)	42
343	23724	523 (2.20)	35 (0.15)	11
337-13	24471	423 (1.73)	91 (0.37)	44
Total			208	97

Number of calli was calculated based on the plated anthers forming calli. Regeneration efficiency was calculated based on the plated anthers forming green plantlets. Number in parantheses are in percentage. DH = double haploid.

Table 4. Resistance of double haploid rice population to three of *Pyricula oryzae* in screenhouse.

Race	No. DH of plants			Total
	Resistant (R)	Medium resistant (MR)	Susceptible (S)	
001	46	37	11	94
033	33	25	36	94
173	79	8	7	94

DH = double haploid. Scoring of blast resistance was based on IRR St Ev.S (IRRI 1996.

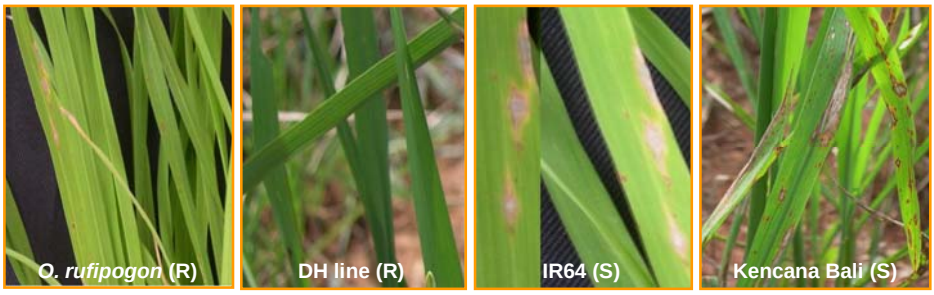

Figure 2. Blast performance on for rice genotypes in a screenhouse. R = resistant, S = susceptible.

haploid plants with good resistance to blast were planted in the field in evaluation of their resistances to blast and the in yield potential.

CONCLUSIONS AND RECOMMENDATION

Out of nine genotypes from three selected families BC_2F_3 of interspesific crossing IR64 x *O. rufipogon*, Calli from three genotypes 149-16, 343, and 337-13 showed most responsive in producing green plantlet after cultured on R_1 (MS + 0.5 mg/l NAA + 2 mg/l kinetin + 40 g/l sucrose + 0.16 g/l putrecine) regeneration medium.

Among 208 green plantlets, 42 double haploid (DH) plants were derived from genotype 149-16, 11 DH plants derived from genotype 343, and 44 DH plants derived from genotype 337-13.

Among 282 DH plants tested for blast resistance in the screenhouse, 46 plants, 33 plants, and 79 plants were resistant to race 001, 033, and 173, respect-ivelly. Twenty eight plants and the wild rice *O. rufipogon* were resistant to the three races.

The double haploid plants with good resistance performance need to be evaluated further in the field to confirm their resistance to blast disease and to evaluate the in yield potential.

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