

Anther Culture of BC₁F₁ (KDML105//IRBB5/KDML105) Hybrid to Produce Bacterial Blight Resistance Doubled Haploid Rice

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ABSTRACT

Maltose was found to be a better carbon source for callus induction in BC₁F₁ (KDML 105//IRBB5/KDML105) anther culture compared with sucrose. Statistical analysis, however, showed that increasing maltose or sucrose concentrations had no differential promotive effects on callus formation. One-step plantlet formation was found when maltose and NAA were supplemented together in the induction media. Adding 2 mg/l 2,4-D to the medium further increased the percentage of callusing anthers from 5.57% to 10.19%. However, the highest percentage of green plant regeneration was obtained (1.29%) from calli induced on N₆ medium without 2,4-D and subsequently cultured on regeneration medium containing MS supplemented with 2 mg/l BAP, 0.2 mg/l NAA, 300 mg/l casein hydrolysate, 15% coconut water, and 30 g/l sucrose. AFLP analysis of all six anther-derived plants showed 57.3% to 67.12% recurrent parental alleles. After planting, seeds were detected in two out of six anther culture-derived plants indicating the occurrence of spontaneous chromosome doubling in these plants. Unfortunately, none of these six plants contained bacterial blight resistant gene (*xa5*) as detected by specific PCR-based RG556 marker and pathogen inoculation.

Key words: KDML 05, anther culture, maltose, 2,4-D, AFLP, RG556, bacterial blight

INTRODUCTION

The production of haploid plants and doubled haploid plants from anther culture offers a rapid achievement of homozygous lines for early release of new crop varieties. Many desirable traits such as high grain weight, disease resistance, dwarf plant type and abiotic stress tolerance were introgressed into rice breeding population by culturing of anthers. Unfortunately, low percentages of both callus induction and plant regeneration are the principal constraints in

establishing successful anther culture in some rice varieties especially in indica rice since these critical culturing responses are genotype dependent (Roy and Mandal, 2005). Consequently, the effective culture medium used for some rice varieties may not be appropriate for others, and the composition of culture media should be carefully selected when the anthers of particular rice variety was subjected to culture.

Sucrose is generally added in rice anther culture media to serve as the standard carbon source and the osmotic regulator. However, many

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reports revealed that the percentages of callus induction and plant regeneration could be increased by using maltose instead of sucrose (Lentini *et al.*, 1995). In addition, the type of auxin in anther culture medium has been proposed to regulate the formation of rice callus. It was found that culturing on 2,4-D supplemented-media stimulated callus induction and cell proliferation in rice anther culture whereas having NAA resulted in direct androgenesis (Ball *et al.*, 1993).

This work aimed at investigating the effects of maltose, sucrose and 2,4-D on the anther culture response of the BC₁F₁ hybrid of two recalcitrant genotypes, Khao Dawk Mali 105 (KDML105) a well-known aromatic rice variety and IRBB5 a bacterial blight resistant rice variety containing *xa5* resistant gene. In addition, the regeneration ability of the anther calli was also observed. After anther culture-derived plants (AC-derived plants) were obtained, the Amplified Fragment Length Polymorphism (AFLP) was used to assess the contribution of the two parental genomes in these plants, and subsequent detection of *xa5* resistant gene for bacterial blight resistance was done using PCR-based marker.

MATERIALS AND METHODS

BC₁F₁ seeds culturing and panicles collection

BC₁F₁ seeds (KDML105//IRBB5/KDML105) were obtained from the crossing between KDML 105 (a bacterial blight susceptible variety) and IRBB5 (the donor parent containing *xa5* bacterial blight resistant gene). The BC₁F₁ seeds were cultured on a modified MS medium (Murashige and Skoog, 1962) supplemented with 2-3 mg/l BAP, 1 g/l yeast extract, 15% coconut water, 30 g/l sucrose and 0.8% agar. The BC₁F₁ plants having *xa5* gene were selected by PCR-based RG556 marker (Huang *et al.*, 1997). Healthy tillers from the selected BC₁F₁ plants were separated and grown in pots. The panicles were collected from the primary, secondary and also

tertiary tillers of plants when the microspores in anther were at the mid-to-late uninucleate stage as seen from the distance between the auricles of the two last leaves that reached 6-12 cm.

Anther culture

The panicles covered with flag-leaf sheaths were wrapped in a moist-soft paper, sealed in a plastic bag, and kept in the dark for 8-10 days at 12°C. After this cold-pretreatment, the panicles were surface-sterilized by spraying with 70% ethanol and the flag-leaf sheaths were removed. Then the anthers were cut off and cultured on callus induction media containing N₆ salts and vitamins (Chu, 1978), supplemented with 2 mg/l NAA, 1 mg/l kinetin, 500 mg/l casein hydrolysate, 0.7% agar and also different concentrations of maltose or sucrose (40, 50, 60 g/l) at the adjusted pH of 5.8. The cultures were maintained under alternate 16/8 h light/dark at 25 ± 2°C for 45-50 days. The numbers of anther calli formation were recorded and the percentages of calli induction were calculated. The experiment was set as 2×3 factorial in completely randomized design with three replications.

An addition of 2 mg/l of 2,4-D to callus induction medium of the same formular described above having 50 g/l maltose was also performed to determine the effect of 2,4-D on callus induction.

Callus differentiation

Anther calli of 1-2 mm diameter were randomly collected and transferred to the regeneration media consisted of MS salts and vitamins, supplemented with different concentrations of kinetin (1, 2, 3 mg/l), 300 mg/l casein hydrolysate, 1 g/l L-proline, 15% coconut water, 30 g/l sucrose and 2.5 g/l phytagel. An addition of regeneration medium having 2 mg/l BAP and 0.2mg/l NAA to replace kinetin and L-proline was set. The cultures were kept under alternate 16/8 h light/dark at 25 ± 2°C for 15-20

days. The numbers of calli producing complete plantlets were recorded and the percentage were calculated.

AFLP analysis

To assess the contribution of two parental genomes in AC-derived plants, AFLP was performed as described by Vos *et al.* (1995) with some modification. Fifteen combinations of primer (synthesized by KU Vector), set E primer (*Eco*RI end) and set M primer (*Mse*I end), were used. Only clear AFLP bands were scored as present or absent. AFLP fingerprints of KDML105, IRBB5 and also AC-derived plants were analyzed.

Detection of *xa5* resistant gene for bacterial blight resistance by PCR-based marker

The DNA of the resistant donor parent (IRBB5), recurrent susceptible parent (KDML105), and AC-derived plants were extracted from the leaves using the method described by Agrawal *et al.* (1992) and subjected to PCR amplification using synthesized primers (KU Vector). The RG556 primer linked to the *xa5* resistant gene was used to detect the presence of resistant gene. The sequence of RG556 F is 5' TAGC TGCTGCCGTGCTGTGC 3' while RG556

R is 5' AATATTTTCAGTGTGCATCTC 3' (Huang *et al.*, 1997). PCR products were digested with *Hpy* CH4 IV restriction enzyme to detect the polymorphic DNA bands from bacterial blight resistant and susceptible plants (Sanchez *et al.*, 2000).

RESULTS AND DISCUSSION

Anther culture

Ten days after incubation on callus induction media, approximately 90% of anthers turned brown (data not shown). This result, however, was not surprising because Guzman and Zapata-Arias (2000) also reported this changing of anther colors which is possibly due to the transition of gametophytic phase to sporophytic phase during androgenesis. In addition, our results showed that anther calli asynchronously emerged through the split lobes of these browning anthers after 45-50 days of culturing (Figure 1A). Most of the responding anthers produced multiple calli which ultimately became yellowish in color having both compact and friable callus types (Figure 1B). The calli were all in satisfactory condition. This is the first report on anther culture of BC₁F₁ seeds (KDML105//IRBB5/KDML105).

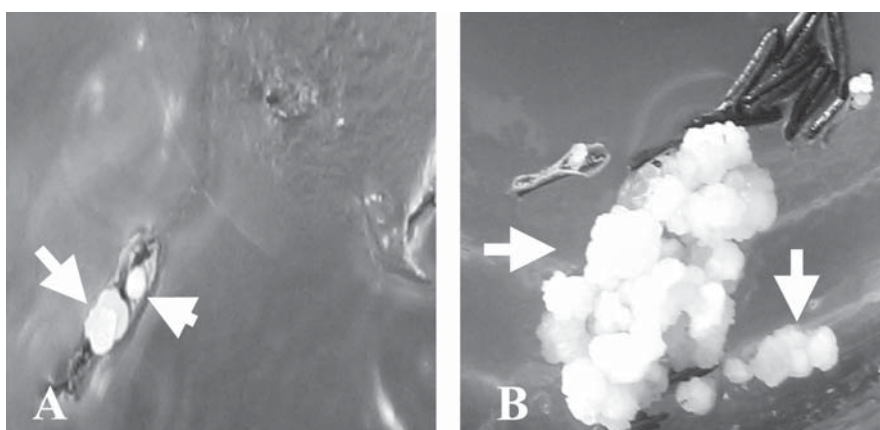


Figure 1 Calli formation: (A) calli emerged through the split lobes of anther (arrow), (B) the anther calli (arrow), after 45-50 days of culturing on callus induction media containing maltose.

The effects of maltose and 2,4-D on callus induction

Using different concentrations of matose and sucrose in the culture medium, it was found that the percentages of calli induction ranging from 4.61% to 6.11% in maltose but only 3.33% to 3.43% in sucrose (Table 1). Callus formation was significantly affected by the type of sugar ($P=0.01$) but not significantly affected by the concentration of sugar itself. The highest percentage of callus induction was obtained by culturing BC₁F₁ anthers on N₆ medium supplemented with 2 mg/l NAA, 1 mg/l kinetin, 500 mg/l casein hydrolysate, 60 g/l maltose and 7 g/l agar. Maltose, therefore, seemed to be a preferred carbon source for prolific callus

formation in rice anther culture as also reported by Lentini *et al.* (1995). The beneficial effect of maltose has been ascribed to its slow degradation which results in stabilization of medium osmolarity (Kuhlmann and Foroughi-Wehr, 1989). In contrast, sucrose is rapidly hydrolysed to glucose and fructose, thereby, the osmolarity of the medium became double causing the negative effect on callus formation (Xie *et al.*, 1995).

Although callus induction rate gradually increased as the concentration of maltose or sucrose increased (Table 1), statistical analysis showed that rising of sugar concentration (40, 50, 60 g/l) had no differential promotive effects on callus formation. This result did not agree with

Table 1 Effects of maltose, sucrose and 2,4-D on anther callus formation of BC₁F₁ (KDML105//IRBB5/KDML105) hybrid.

Types of sugar in callus induction medium	Concentration of sugar (g/l)	Number of cultured anthers	Number of callusing anther	Percentage of callus induction (%)
Sucrose	40	1,140	38	3.33
	50	1,808	62	3.43
	60	848	29	3.42
Maltose	40	4,252	196	4.61
	50	9,979	557	5.57
	60	7,695	470	6.11
	50a	4,378	446	10.19

Analysis of variance: ANOVA table

Source	Df	SS	MS	F(cal) value	F(table) value	
					5%	1%
Treatment	5	22.16				
Factor A	1	18.68	18.68	14.83**	4.75	9.33
Factor B	2	1.69	0.98	0.78 ns	3.88	6.93
A×B	2	1.52	0.76	0.60 ns	3.88	6.93
Error	12	15.12	1.26			
Total	17	37.28				

** indicated highly significance at 1%, ns indicated no significance

50a referred to 50 g/l maltose supplemented medium + 2 mg/l 2,4-D

Factor A was types of sugar, viz. maltose and sucrose.

Factor B was sugar concentration, viz. 40, 50 and 60 g/l.

the report of Ching (1982) showing the increase of sugar concentration (30, 60, 90 g/l) to promote the higher percentage of callusing anthers as well as plantlet formation. The contradictory results may be due to the narrow range of sugar concentrations used which could not cause distinctive effects on callus induction in this study.

It is also interesting to find that some of yellowish compact calli grown in 50 g/l or 60 g/l maltose containing media could differentiate to complete plantlets. The development of complete plantlets while they are culturing on induction medium is known as “one-step plantlet formation”. This result agreed with other reports showing that addition of maltose to NAA containing callus induction media promoted the formation of complete plantlet in rice anther culture (Zhao *et al.*, 1999). Since the formation of plantlets by one step did not frequently occur in anther culture of KDML105 hybrids (Lertvichai, 1995; Boonintara, 2004), three complete green plantlets (2.63%, data not shown) obtained from this experiment is considered a positive and satisfactory result. It should be noted here that genotype of anther donor plants, the type and concentration of sugar as well as the type of auxin have some effect on one-step plantlet formation (Zhao *et al.*, 1999).

To further increase the percentage of callus formation, 2 mg/l of 2,4-D was added to 50 g/l maltose supplemented-medium. The results showed that the percentage of callus formation was increased from 5.57% to 10.19% (Table 1). Although the addition of 2,4-D favoured callus initiation and proliferation, the organogenesis of the calli might be inhibited hence one-step plantlet formation was not obtained. This result did not agree with those obtained by Datta *et al.* (1990) showing the combination of NAA and 2,4-D supplemented in callus induction medium promoted green plantlet formation in rice. This opposing result is possibly due to the powerful influence of genotype of anther donor plants on the response of rice anthers to these auxins.

Callus differentiation

After culturing on regeneration media, differentiation of the calli was observed at 15-20 days. The percentages of calli development are shown in Table 2. The results indicated that the highest percentage of green plantlet formation (1.29%) was obtained when the calli grown on induction media without 2,4-D were transferred onto regeneration medium containing MS salts and vitamins supplemented with 2 mg/l BAP, 0.2

Table 2 Development of calli on regeneration media.

Callus induction media	Regeneration media	Number of cultured anthers	Browning calli (%)	Proliferated calli (%)	Plantlet formation (%)	
					Green plantlets	Albino plantlets
without 2,4-D	SR1	140	14.29	74.29	0.71	4.29
	SR2	123	18.71	65.85	0.81	1.63
	SR3	180	10.00	58.33	0.00	13.89
	MR1	77	9.09	12.89	1.29	12.99
with 2 mg/ 2,4-D	SR1	15	13.33	80.00	0.00	0.00
	SR2	82	2.44	96.34	0.00	12.12
	SR3	93	0.00	92.47	0.00	4.30
	MR1	92	4.34	92.39	1.09	0.00

SR1, SR2, SR3 = MS + different concentration of kinetin (1 mg/l, 2 mg/l, 3 mg/l) + 300 mg/l casein hydrolysate + 1 g/l L-proline + 15% coconut water + 30 g/l sucrose + 2.5% phytagel

MR1 = The same formular as SRs media but having 2 mg/l BAP and 0.2 mg/l NAA to replace kinetin and L-proline.

mg/l NAA, 300 mg/l casein hydrolysate, 15% coconut water, 30 g/l sucrose and 2.5% phytagel (MR1 medium). It was also found that the percentage of green plantlet formation of calli cultured on the media without BAP and NAA was increased when the concentration of kinetin increased. By two steps culturing (callus induction and subsequent regeneration of calli), the total of four green plantlets were obtained (Figure 2A,B). However, one of these plantlets died during subculturing leaving only three plantlets for further investigation.

It is interesting to find that regeneration medium highly supported callus proliferation (80%-96.34%, Table 2) of those previously grown in the medium containing 2 mg/ 2,4-D but the complete plantlets formation was better formed in the media without 2,4-D. These results implied that regeneration response of calli was possibly affected by the interaction between the composition, particularly the types of auxin, of induction media and regeneration media.

Although the percentages of callus induction (3.33-6.11%) and green plantlet formation (0.00-1.29%) of BC₁F₁ in this study were lower than those of Lemont/KDML 105 hybrid (2.22%-44.46% and 0.00%-20%

respectively) as reported by Lertvichai (1995), they were comparable to those of KDML 105/Chainat1 hybrid (Boonintara, 2004). The low outcome of callus formation and recovering plants from anther culture of indica rice are known to be genotypic dependent (Khanna and Raina, 1998).

A large percentage of albinos (more than 90%, data not shown) obtained in this study is considered unsatisfactory but similar to other reports of albinos ranging from 5% to 100% in rice anther culture, especially in indica rice (Bhojwani *et al.*, 2001). Several factors, including pre-treatment, culture medium and culturing steps considerably affected the frequency of albinism. However, high sugar concentration might be another cause of albino plant formation as seen in the increase percentage of albino plantlet of japonica rice (Tainan 5) in the increased sugar culture (Chen, 1978).

AFLP based background analysis in anther culture-derived plants

AFLP analysis was performed on six AC-derived plants (three plants obtained by one-step plantlet formation and the others from two-step plantlet formation) to assess the contribution of two parental genomes in these plants. Out of 373

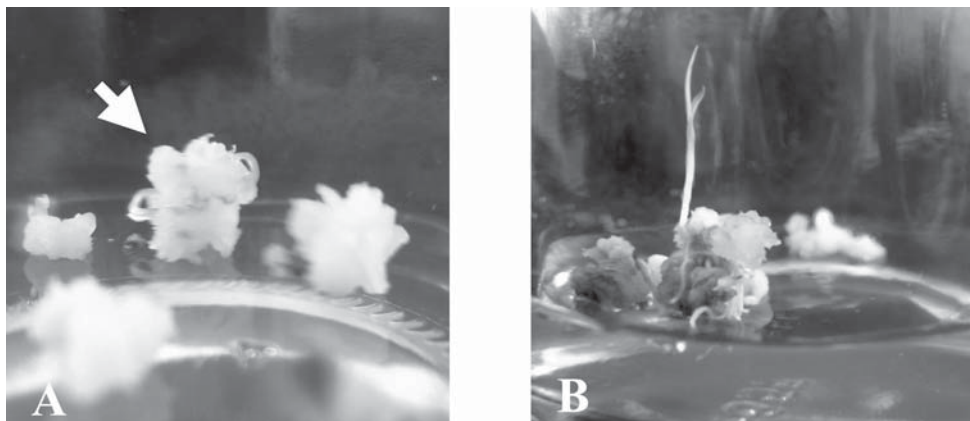


Figure 2 Development of calli: (A) green spot producing callus (arrow), (B) green plantlet regeneration, after 15-20 days of culturing on regeneration media containing MS supplemented with 2 mg/l BAP, 0.2 mg/l NAA, 300 mg/l casein hydrolysate, 15% coconut water, and 30g/l sucrose.

clearly amplified bands generated by 15 primer combinations, 73 bands showed polymorphisms between the parents (Figure 4) of which 50 bands were specific for KDML105 and 23 specific bands for IRBB5 (data not shown). By assuming random distribution of AFLP markers in rice genome, it was found that the percentage of recurrent parental alleles (KDML105) recovered in AC-derived plants population was found ranging from 57.3% to 67.12% (Table 3). This result is somewhat narrower than the anticipated distribution of chromosomes containing recurrent parental alleles (50%-100%) in plantlets obtained from anther culture of BC₁F₁ plants, which is possibly due to the fact that AFLP primers used in the present study did not cover the whole genome. Furthermore, only six AC-derived plants were obtained and could not, thereby, represent the broad distribution of the overall AFLP alleles in the population. In addition, Guiderdoni (1991) reported that androgenesis of microspores containing more genetic make-up of recurrent parent may be masked by gametic selection. This selection resulted in the segregation distortion of alleles and preventing AC-derived plants from being truly

BC₁F₁ gametic array as also shown in the anther culture of japonica/indica and indica/indica rice hybrids.

After planting six healthy AC-derived plants, seeds were obtained in two out of these six plants (33.33%, data not shown) indicating the occurrence of spontaneous chromosome doubling which resulted in two homozygous lines from anther culture of BC₁F₁ (KDML105//IRBB5/KDML105). This result was not surprising since the mechanisms to double chromosome can occur at various stages *in vitro*, including callus formation, callus re-differentiation and embryogenesis in rice anther culture, and even in tillers (Bishnoi *et al.*, 2000). Although the percentage of chromosome doubling obtained from this study (33.33%) was lower than the anther culture of KDML105/RD23 (86.1%) reported by Pakdeechanuan (1997), it was higher than that of KDML105/Lemont (25%) (Lertvichai, 1995) and comparable to those of KDML105/Chainat1 (33.33%) (Boonintara, 2004). Genotypic dependence was suspected to be the main cause affecting the frequency of chromosome doubling in rice anther culture (Sopory *et al.* 1996).

Table 3 The percentage of recurrent parental alleles (KDML105) recovered in AC-derived plants population as detected by AFLP using 15 primer combinations.

Individual AC-derived plants	Number of specific bands presented only in KDML105	Number of specific bands presented only in IRBB5	Percentage of recurrent parental alleles (KDML105) recovered in AC-derived plants (%)
1	49	24	67.12
2*	47	26	64.38
3	42	31	57.53
4*	47	26	64.38
5	45	28	61.64
6	43	30	58.90
Average percentage of recurrent parental alleles recovered in AC-derived plants			62.33

* indicated spontaneous double haploids

Detection of *xa5* resistant gene for bacterial blight resistance by PCR-based marker

Since the anther donor plants (BC₁F₁) were confirmed to be heterozygous for the *xa5* resistant gene (data not shown), AC-derived plants from BC₁F₁ were also tested for the *xa5* gene using PCR-based marker RG556. The PCR product gave monomorphic amplification products of 1,600 bp (Figure 3A). However, after digesting with *Hpy* CH4 IV, polymorphism of DNA bands between resistant and susceptible plants was detected. Two bands of 1,000 bp and 300 bp (doublet) were found in IRBB5 (bacterial blight resistant variety) while non-digested DNA band (1,600 bp) was shown in

susceptible variety of KDML105 (Figure 3B). Genotyping by PCR-based method revealed none of these six AC-derived plants contained the *xa5* gene for bacterial blight resistance (Figure 3B). This result was confirmed by pathogen inoculation test on 45 days old plants grown from healthy seeds of two homozygous lines (obtained by spontaneous chromosome doubling as described in AFLP analysis section) which showed bacterial blight susceptibility (data not shown).

Sanchez *et al.* (2000) reported that the distance between RG556 marker and the *xa5* gene was 0.8 cM, then the loss of the *xa5* gene in six AC-derived plants in the present study possibly

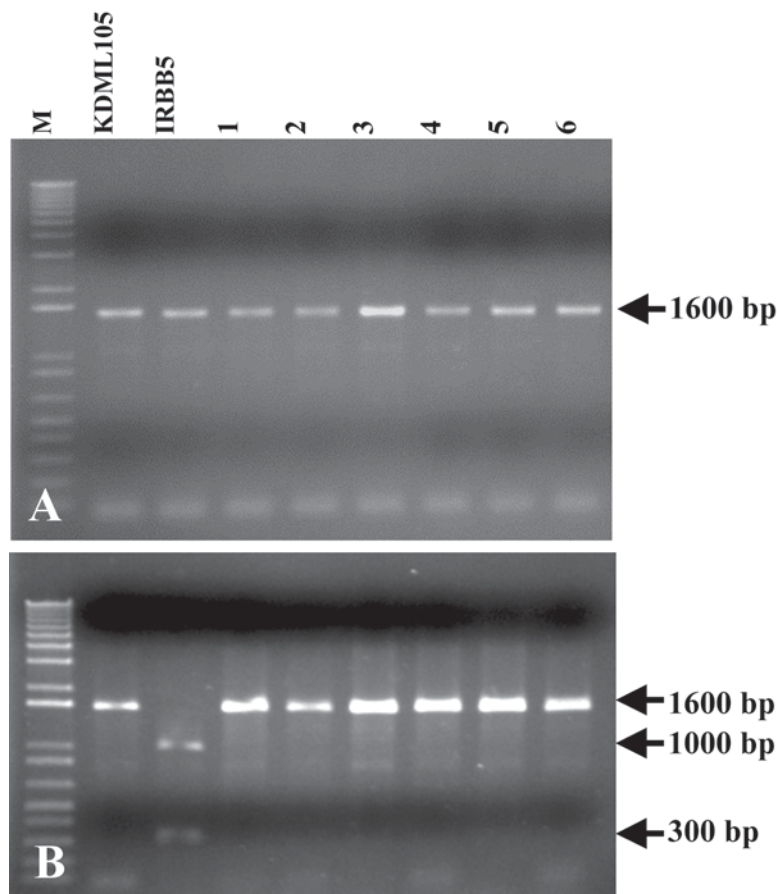


Figure 3 PCR analysis of the bacterial blight susceptible variety (KDML105), resistant variety (IRBB5) and AC-derived plants (the samples number 1, 2, 3, 4, 5, 6): (A) Monomorphic bands amplified with primer RG556, (B) PCR products digested with *Hpy* CH4 IV. M= 1 kb plus DNA marker.

caused by either the recombination of RG556 marker and the *xa5* resistant gene or the segregation of gene during gametogenesis.

CONCLUSION

Two homozygous lines were rapidly achieved by anther culture of BC₁F₁ hybrid (KDML105//IRBB5/KDML105) and subsequent spontaneous chromosome doubling. In the present study maltose has proven to be a preferred carbon

source compared to sucrose as seen from the significant effect on callus formation. Furthermore, one-step plantlet formation was promoted when callus induction medium supplemented with combination of maltose and NAA was used. Although the percentages of callusing anther and callus proliferation were increased by adding 2,4-D to NAA containing induction medium, the percentages of organs formation as well as complete plantlet formation were very low, moreover one-step plantlet formation did not

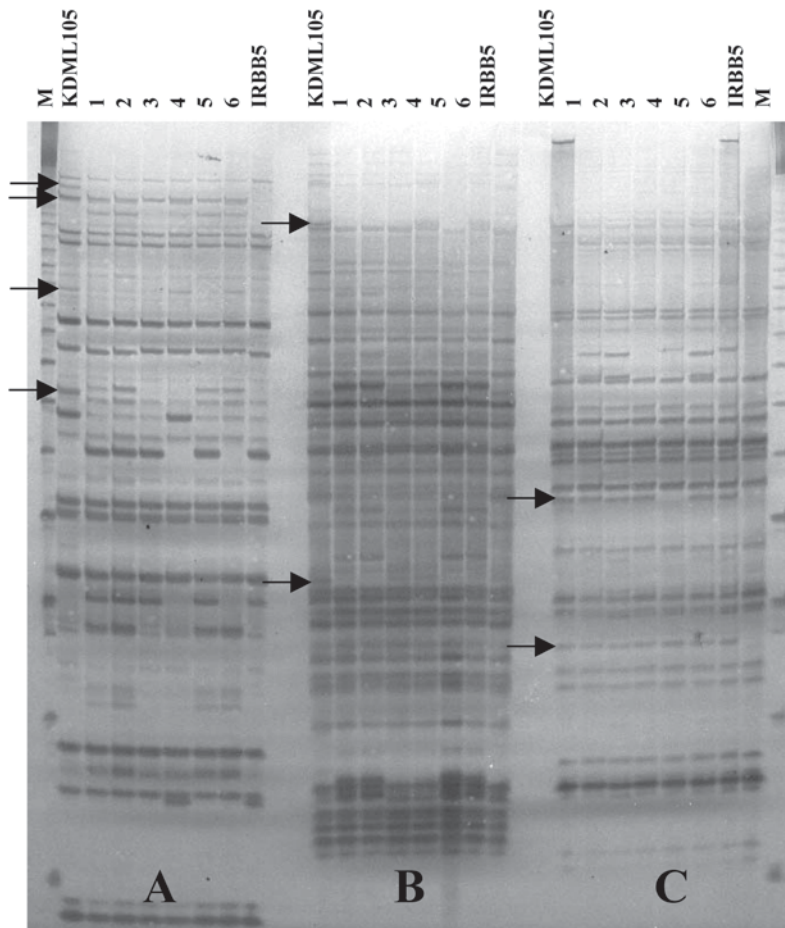


Figure 4 AFLP fingerprint of the bacterial blight susceptible variety (KDML105), resistant variety (IRBB5) and AC-derived plants (the samples number 1, 2, 3, 4, 5, 6) generated by different primer combinations: (A) E-AGG / M-CTC primer (B) E-AAG / M-CAA primer (C) E-AAG / M-CAG primer, M = 25 bp DNA size marker (Life Technologies); arrows indicate polymorphic bands specific for KDML105.

occur. By two-steps culturing, the highest percentage of green plant regeneration was obtained (1.29%) from calli induced on N₆ medium without 2,4-D and subsequently cultured on regeneration medium containing MS supplemented with 2 mg/l BAP, 0.2 mg/l NAA, 300 mg/l casein hydrolysate, 15% coconut water, and 30g/l sucrose. The contribution of recurrent parental genome in AC-derived plants was revealed by AFLP analysis. Even though these AC-derived plants did not contain a bacterial blight resistant gene (*xa5*) when screened by PCR-based RG556 marker, other desirable traits such as dwarf plant type, photoperiod insensitive response and aroma, characteristic of the parents could be obtained from them.

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