Evaluation of callus induction and plant regeneration on different media in rice F1 hybrids using Anther culture

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Abstract

Anther culture has become a powerful tool for the rapid production of haploid and inbred lines used for obtaining hybrid cultivars. The genotype and the composition of the callus induction basal medium were the major determinants of regeneration response. It is important to investigate appropriate media for combinations with high callus induction frequency and green plant regeneration. Callus induction ability of different media on F1 hybrid Paw San Hmwe (PSM) x IR 24 and plant regeneration ability on different media of PSM x IR 24 were evaluated through anther culture. The callus induction frequency was higher on SKI media due to might be lower level of $NH4^{+}$. The frequency of callus induction was higher on SKI and He 2 media for Japonica x indica (PSM x IR24). In the present study, it was observed that green plant frequency was found on SKI and He2 and there was no green plant frequency on N6 medium. Our result indicated that SKI medium has the best response in anther culture ability and plant regeneration ability and SKI medium should be exploited in anther culture is called double haploid breeding program.

Index Terms: Anther culture, Media, PSM x IR 24, Callus induction, Plant regeneration

1. INTRODUCTION

Rice (*Oryza sativa* L.) is one of the most important cereal crops cultivated in the world. It provides food for more than half of the world population (Sasaki, 2005). Plant breeders used conventional methods such as hybridization, selection, mutation, etc, to produce new rice varieties. The conventional plant breeding methods can be achieved by combining the desired traits through crossing with another desired character .Plant breeders attempt to improve rice by using biotechnology. Therefore, plant tissue culture has become an important tool for breeding improvement in rice (Ge *et al*., 2006). Among plant tissue culture techniques, anther culture is called double haploid breeding is the simplest and more efficient method (Niizeki and Oono 1968).

Double Haploid Breeding (DHB) in rice breeding are shortening the breeding cycle by immediate fixation of homozygosity, increase selection efficiency, widening genetic variability through the production of gametoclonal variants, and allowing the early expression of recessive genes.

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DHB has become a powerful tool for the rapid production of haploid and inbred lines used for obtaining hybrid cultivars (Sopory and Munshi, 1996) and it has reduced the time required for breeding new cultivars by at least 3 to 5 years (Tai, 2003). In order to get pure line rapidly, DHB method should be used in breeding program.

In DHB, doubled haploids are usually raised from F1 plants. Hence, after getting F1 hybrids, DHB method could be used. Inoculation of anthers in five basal media viz. N6, modified N6, R3, He2 and He5 each one supplemented with NAA (2 mg /1), kinetin (0.5 mg /1) and sucrose (5%) showed that the rate of callusing was highest (8.3%) in He2 medium (Mandal and Gupta, 1997). Reported the genotype and the composition of the callus induction basal medium were the major determinants of regeneration response, also revealed a significant interaction between the media used for de-differentiation (callusing) and re-differentiation (plantlet regeneration). Therefore, it is important to investigate appropriate media for combinations with high callus induction frequency and green plant regeneration.

In Myanmar, although many efforts have been made for rice improvement, they are taken under natural selection, conventional breeding and mutation breeding. Application of double haploid breeding in rice breeding has very rarely done in Myanmar. Effective use of double haploid breeding based rice breeding is needed to explore to upgrade rice production compared to conventional breeding for Myanmar. For these reasons, the experiment

was conducted in Japan having advanced double haploid based rice breeding with the following objectives;

(1) to examine callus induction ability of different media on F1 hybrid Paw San Hmwe (PSM) x IR 24
(2) to evaluate plant regeneration ability on different media of PSM x IR 24 through anther culture

2. RESEARCH METHOD

The experiment was conducted at the laboratory of Tropical Crop Science in Tokyo University of Agriculture from July, 2016 to August, 2017. Paw San Hmwe (Japonica) and IR 24 (Indica) was selected parental lines for hybridization. It was grown and cross of japonica x indica were conducted to obtain F1hybrid. Paw San Hmwe x IR 24 was used to select appropriate media for anther culture.

Anther Pre Treatment

Panicles of F1 hybrid plant (PSMx IR24) were collected in the morning (9 am to 10 am) and harvested when the flag leaf had just emerged. Microspores conditions were at the early to mid uninucleated stage as observed by 1% acetocarmine staining. Panicles were wrapped in muslin cloth, sealed in polythene bags and 10 panicles from F1 plant were cold pretreated at 10.C for 7 days in the dark. After cold treatment, panicles were surface sterilized with 70% ethyl alcohol, washed in sterile distilled water, kept for 2-3 min in 0.1% HgCl2 solution and then again washed 4-5 times with sterile distilled water.

Anther Culture

The upper one-third of the spikelet was cut off and the anthers gently squeezed out with a forceps. An average of 5-6 anthers per test tube was dusted in the induction three media, N6, SKI and He2 and cultured. 12 test tube was constituted one replicate and an average of 4 replicates was cultured for each treatment. The test tube was sealed with parafilm. The cultures were kept at 27.C for 4- 10 weeks in the dark for callus induction. Calli of at least 1-2 mm diameter were transferred to regeneration media (MS) and subsequently transferred to the culture room at 16 hour light and 8 hour dark regime for plantlet regeneration.

Callus induction

The anther in which developed at early uninucleate to early binucleate stage were cultured on N6, SKI and He2 media with supplemented 2 mg.L-1 2,4-D and 0.5 mg.L-1 Kinetin for callus induction. Cultured anthers were incubated in dark condition at 27.C till callus induction. The anther culture response was observed for callus formation.

Plant regeneration

Calli (1-3 mm) were precultured on 'M' shaped-paper bridges with MS liquid medium (Murashige and Skoog, 1962) with 2 mg.L-1 2,4-D and 0.5 mg.L-1 Kinetin. After 2 weeks, the calli were transferred onto the MS medium with supplemented 1 mg.L-1 NAA, 1 mg.L-1 IAA,1 mg.L-1 BAP and 2 mg.L-1 kinetin. The regenerated calli were observed till green plant formation under 16/8 light/ dark hours at 25 ± 2ºC.

Three basal callus induction media compsition

N6, SK1, He2 media were used for callus induction. Three media compositions were given in table . Anthers were dusted in these plated three media and kept it into BOD incubator for 27°C in the dark condition (Chen *et at*., 1991).

Table : Callus induction media composition

Components (mg/L)	N6	SK1	He2
	Macro(mg/L)		
(NH4)2 SO4	463	231	231
KNO3	3535	3180	3181
KH2PO4	400	540	800
NaH2PO4 H2O	0	0	0
MgSO4 7H2O	185	185	3.5
CaCl2 2H2O	166	440	166
	Micro (mg/L)		
H3BO3	1.6	6.2	1.6
MnSO4 4H2O	22.3	22.3	22.3
ZnSO4 7H2O	8.6	1.5	1.5
Na2MoO4 2H2O	0.25	0.25	0.25
KI	0.8	0.8	0.8
CuSO4 5H2O	0.025	0.025	0.025
CoCl2 6H2O	0.025	0.025	0.025
FeSO4 7H2O	27.8	27.8	27.8
Na2EDTA	37.2	37.5	37.5
	Organic compounds (mg/L)		
Thiamine-HCl	2.5	2.5	10
Nicotinic acid	2.5	2.5	0.5
Pyridoxine-HCl	2.5	2.5	0.5
Glycine	2	2	2
Inositol	100	100	100
2,4-D	20	1	1
NAA	20	1	1
Kinetin	21.1	0.5	0.5
Maltose	3	30	30
Gelrite	2700	2700	2700
Yeast Extract	100	0	0
Casein Hydrolysate	0	200	200
AgNO3	0	8	8

Table: Composition of MS media used

Components	Amount (mg/L) present in Standard medium
Inorganic compound	
	Macronutrients
KNO3	95000
NH4NO3	82500
CaCl2.2H20	8800
MgSO4.7H20	7400
KH2PO4	8500
	Micronutrient
MnSO4.4H20	845
ZnSO4.7H20	430
H3BO3	310
KI	41.5
Na2MoO4.2H2O	12.5
CoCl2.6H20	1.25
CuSO4.5H20	1.25
	Iron Source
Na2.EDTA	3720
FeSO4.7H20	2780
Organic Compounds	**Vitamins**
Myoinositol	2000
Nicotinic acid	10
Pyridoxine HCL	10
Thiamine HCL	20
	Amino acid
Glycine	40

Data analysis: The experiment wasconducted to analyze callus induction and green plant regeneration percentage in Completely Randomized Design (CRD).

3. Result and Discussion

In this study, my investigation was given to callus induction and green plant regeneration through double haploid breeding. Response of PSM x IR 24 to three different media was investigated. Two types of callus induction were found in this study. The embryonic calli (milky form) and non embryonic calli (watery form) were observed within 4-8weeks.

Effect of different media on callus induction from anthers of selected F1 hybrids (PSM x IR 24)

Days to callus formed (days)

Mean days to callus formed on different media cultured were presented in Figure 1. Callus induction started at four weeks of culture and callus induction was observed in the tested media (Plate 1a).

Days to callus formed from anther culture on SKI showed latest mean value of (57days). Among the tested media, SKI media was later than He2 (49days) and N6 (39days). Days to callus formed on N6 media were later than He2 media. In this study, N6 media showed the earliest callus formation.

Lentini et al. 1995 reported that only one out of 35 indica cultivars exhibited pollen callusing on N6 medium. Guha- Mukerjee reported that only 5 out of 18 indica cultivars showed pollen callusing and callus from only one cultivar differentiated into plants on N6 media. The present study was observed like the previous study. These findings were consistent with previous reports (Kaushal et al., 2014).

According to the data, it was observed that among the tested media, SKI medium should be used for callus formed on F1hybrid (PSM x IR 24).

Number of callus formed (no.)

The number of callus formed was observed in 3 tested media which showed highly significant difference (Figure 2). The maximum number of callus formed was found in SKI medium with (13), followed by He2 medium (9) and N6 had minimum number of callus formed (6). Number of callus formed in SKI medium was higher than those of N6 and He2. Number of callus formed on N6 was significantly lower than those of SKI and He2 media. In the present study, callus formed is an important character contributing to green or albino plants. Chu et.al, 1975 observed that the widely used N6 medium for anther culture was found less suitable for Japonica/indica rice anther culture.

Frequency of callus induction (%)

Frequency of callus induction (%) on different media with F1 hybrid (PSM x IR 24) incubated during 2017 at Laboratory of Tropical Crop Science was presented in Figure (3).

There were differences among the tested media on PSM x IR24.

In this study, the medium was supplemented with various concentrations of different growth hormones for the callus induction from anthers. Though callus formation from anthers was observed in all treatments, the highest callus induction frequency was found for SKI (18%), followed by He2 (12%) and N6 (5%). Frequency of callus induction (%) on SKI and He2 media was not different from one another while frequency of callus induction on SKI and He2 media was higher than that of N6 medium. According to the result of this study, the callus induction frequency was higher on SKI media due to might be lower level of $NH4^{+}$ whereas the superior callus induction response was found on He2 medium might be due to containing less amount of MgSO4, (NH4)2SO4 and KH2PO4 which need for callus induction than N6 medium. Silva and Ratnayake , 2009 found that SKI medium was the best medium for callus induction.

Mandal and Gupta (1997) reported that higher callus induction was obtained from He 2 medium among five different media such as N6, modified N6, R3, He2 and He5. In this study, frequency of callus induction was higher on SKI and He 2 media for Japonica x indica (PSM x IR24).

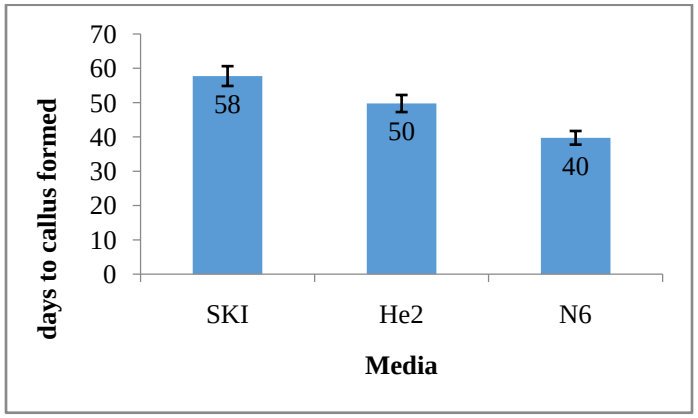


Figure 1 Days to callus formed affected by three different media on PSM x IR 24

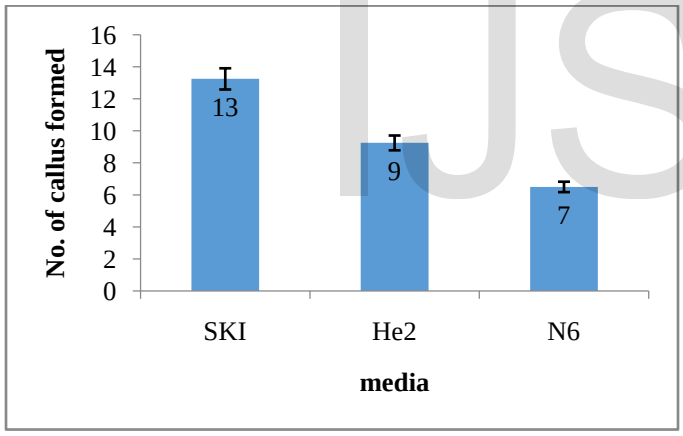


Figure 2 Number of callus formed affected by three different media on PSM x IR 24

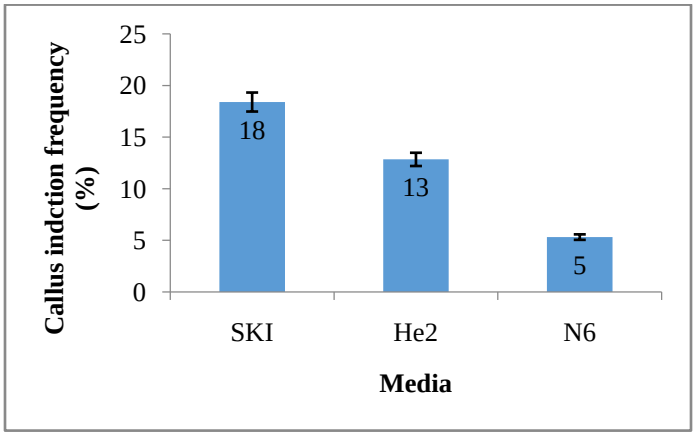


Figure 3 Frequency of callus formed by three different media on PSM x IR 24

Effect of different media on green plant regeneration from anther derived calli

Days to calli that regenerated green or albino plants (days)

The green plant regeneration or albino plants started after two weeks from transferring of different callus induction media to MS media (Table 1). Some calli was differentiated into green plant (Plate 2) or abino plants (Plate 3).

The earliest green plant regeneration was found in SKI (12 days) and followed by N6 (11 days) and He2 (9) . Days to green plant regeneration on SKI was not significantly different from those of N6 and He2. Days to albino plants was also observed on He2 medium (12 days), followed by SKI (9 days) and N6 media (8 days). Days to albino plants formed on different media were not significantly different with each other.

Number of calli that regenerated green or albino plant (no.)

The number of calli that regenerated green or albino plant was observed in 3 tested media which showed highly significant difference (Table 1).

The maximum number of calli regenerated green plant was found on SKI media with (3), followed by He2 (1) and He2 had minimum number of callus formed (0.5). Number of calli that regenerated green plant on SKI medium was higher than those of N6 and He2 while N 6 medium was not different with He2 medim. But in SKI media, albino plant regeneration from calli initiated on SKI

(5) media was high and number of calli that regenerated albino on N6 and He 2 were not different from each other. In this study, number of calli that regenerated green plant was high on SKI medium might be due to presence of AgNO3 which had positive effect on embryogenesis by blocking the effect of inhibitor in culture and it is anti ethylene agent to delay anther response. Lentini et al. (1995) found that AgNo3 increase pollen callusing frequency and green plant regeneration number. Similar reported that AgNO3 had positive effect in anther culture of brassica and wheat (Ghameni et.al., 1994). According to the result of this study, SKI media is the best for green plant regeneration on japonica x indica.

Green plant frequency (GPF) % or Albino plant frequency (ABF) %

The green plant frequency from the calli initiated on three different media was presented on Table (2).

The green plant frequency was observed on SKI (8%), He2 (7%) and N6 (0%). The green plant frequency on different media was not different from one another. The frequency of albino plant was high on these three different media and the values varied 14% on SKI, 14% on He 2 and 8% on N6. Asaduzzaman, et al., 2003 reported that the albino plants regeneration was a major problem in rice anther culture especially in Japonica/indica rice. Roy and Mandal (2005) reported that green plant regeneration is very low and low anther culture response, high albino plant percentage are the critical problem in establishing successful double haploid breeding in rice. In the present study, it was observed that green plant frequency was found on SKI and He2 and there was no green plant frequency on N6 medium. Although green plant frequency was observed on different media apart from N6 medium, albino plant frequency of PSM x IR 24 on three different was higher than green plant frequency. Talebi et.al., 2007 reported that the frequency of albinos might vary from 5% to 100%. The albino development is inversely proportional to green plant regeneration (Kaushal, 2014).

Table 1 Effect of different media on green plant regeneration from anther derived calli

Media	Days to calli that regenerated green plant	Days to calli that regenerated albino plant	Number of calli that regenerated green plant	Number of calli that regenerated albino plant
SKI	12	12	3	5
He2	11	9	1	2
N6	9	8	0.5	0.5

Table 2 Effect of different media on green plant regeneration from anther derived calli

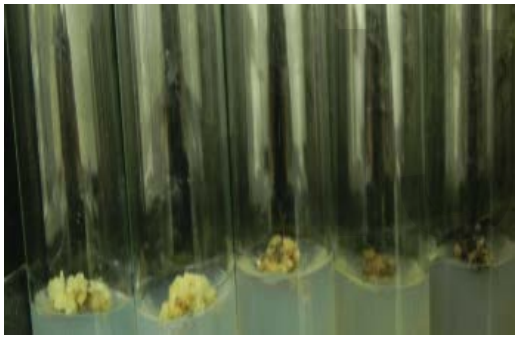
Media	Green plant frequency (GPF) %	Albino plant frequency (ABF) %
SKI	8%	14%
He2	7%	14%
N6	0%	8%

4. Conclusion

F1 hybrid (PSM x IR) was studied to examine callus induction and plant regeneration ability of different media (SKI, He2 and N6) through double haploid breeding method. In this study, almost tested media produced callus. It was concluded that among the tested media, SKI and He 2 media produced highest callus induction on Japonica x indica (PSM x IR24). Although callus induction was high on SKI and He2 media, days to calli formed of SKI and He 2 media was later than that of N6 medium. N6 medium was the earliest callus induction medium in this study and SKI and He 2 media were appropriate media on Japonica x indica for callus induction.

The regenerated plant and albino plant were found in different days on different media. But days to regenerated plant or albino plant of PSM x IR 24 on different media were not significantly different from one another. For green plant regeneration, SKI medium produced highest on Japonica x indica and SKI medim is suitable among the tested media.

It was concluded that the efficient establishment of callus induction and plant regeneration ability through double haploid breeding was greatly facilitated by the callus induction media. In addition, F1 hybrid (PSM x IR 24) was identified as the efficient cross for inducing calli. Therefore, SKI medium was selected as top performance medium for japonica x indica (PSM x IR 24) because of higher inducing calli and green plant regeneration compared to other media. This information might be helpful for rice improvement program especially production of new rice varieties. The successful production of double haploid in rice depends on not only media but also genotype. In this study, we have found that the best callus frequency and plant regeneration frequency was seen at SKI media on PSM x IR 24. Therefore, SKI medium has the best response in anther culture ability and plant regeneration ability and SKI medium should be exploited in anther culture is called double haploid breeding program.



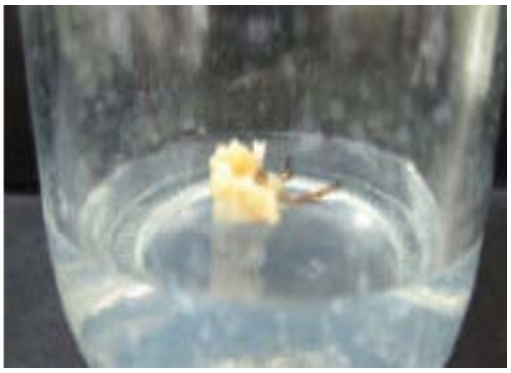


Plate 1 The embryonic calli induction of PSM x IR 24

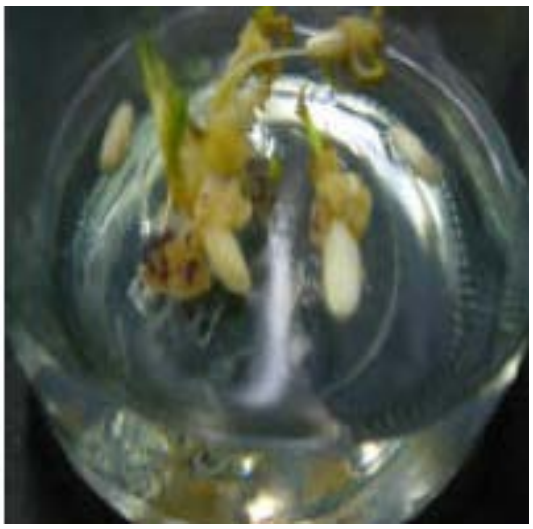


Plate 2The green plant regeneration of PSM x IR 24 The albino plant of PSM x IR 24

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