Sarhad J. Agric. Vol. 23, No. 1, 2007

TISSUE CULTURE TECHNIQUES FOR CALLUS INDUCTION IN RICE

Hidayat Ullah*, Iltaf Ullah*, Sultan Akbar Jadoon* and Hamid Rashid**

ABSTRACT

Seeds of two rice varieties Basmati-370 and Basmati-385 were evaluated for invitro callus induction at Agricultural Biotechnology Institute in National Agricultural Research Center (NARC), Islamabad during 2001-02, using M.S and N6 media supplemented with 2, 4-D (2,4-Dichlorophenoxi acetic acid) and BAP (6-benzylaminopurine) at @ 2.0, 2.5 and 0.1, 0.5 mgl^{-1} respectively. Objective of our study is to know the actual procedure for seed sterilization and to provide a well-adopted technique for callus induction in these varieties by using M.S and N6 media along with different concentrations of growth hormones. Seeds were used as explant source and to avoid chance of contamination sodium hypochlorite and ethanol at the rate of 50% and 70% were used respectively. Best response toward callus induction were observed for Bas-385 on both MS and N6 media however N6 media was proved to be best. Calli obtained from Bas-385 were friable and vigorous as compare to Bas-370. Bas-370 respond moderately on MS and N6 media at various combinations of 2,4-D and BAP. Bas-370 on MS media supplemented with 2,4-D and BAP @ 2.0 and 0.1 mgl^{-1} gave good performance towards callus induction. Over all results indicated that best callus were induced on MS and N6 when supplemented with 2,4-D and BAP @2.0 and 0.0 mgl^{-1} for variety Bas-385.

INTRODUCTION

In Pakistan rice is the second leading crop after wheat. In 2000-2001, the world annual production of rice was 598 million tones (FAO 2002). Asia is the largest producer of rice, with Bangladesh, China, Philippines, Thailand and Vietnams as the leading rice producing nations. In Pakistan rice covered an area of 2.52 million ha, with the production of 5.16 million tones in 1999-2000. It also play important role in our national economy. During 1999-2000 about 1.92 million tones of rice was exported and earn about 465.8 million US$.

Rice is susceptible to a range of diseases and pests, which annually destroy about 55 percent of rice crops. The most common diseases are caused by the fungi sheath blight and rice blast, and the stalk borer is a common insect pest. Rice is composed of essential food components, therefore more than two billions people in the globe depend on rice for more than half of the proteins and calories they consume (Khan *et al*. 2000). Due to its increasing importance in nutrition and economy, it is now felt that new varieties of rice, having good agronomic characters, should be evolved.

Crop improvement through tissue culture techniques is easier and more often in use as compared to conventional plant breeding (Yamada, 1986). Somaclonal variations commonly appear after tissue culture, which involve a callus stage (Larkin and Scow Croft 1981). Callus is undifferentiated mass of rapidly proliferating cells, can be obtained by culturing explants source (seed, node, bud, leaves, meristem and root tips etc) on nutrient medium containing specific growth regulators along with a standard recipe of chemicals. Rashid *et al.* (2000) studied that rice seeds have more potential for callogenesis as compared to node or tip. Successful callus induction from rice seed has been reported by several researchers (Gonalz 2000; Navraj *et al*. 1999; Marrassi 1996; Valdez *et al*. 1997; Xie *et al*. 1995). But an improved method for callogenesis was reported by Rashid *et al.* 2000).

The present study is based on tissue culture techniques carried out in ABI laboratory for callus induction in rice varieties viz. Basmati-370 and Basmati-385. Two types of basal media M.S (Murashige and Skoog 1962) and N6 (Nitsch and Nitsch, 1969) supplemented with 2,4-D (2,4-dichlorophenoxyacetic acid) alone or in combination with different concentrations of BAP (6-benzylaminopurine) were used for callus induction.

* Department of Plant Breeding and Genetics, NWFP Agricultural University, Peshawar – Pakistan.
** Agricultural Biotechnology Institute, National Agricultural Research Center, Islamabad – Pakistan.

MATERIALS AND METHODS

The research work for callus induction in rice was conducted at the tissue culture laboratory of Agricultural Biotechnology Institute in National Agricultural Research Center (NARC), Islamabad, during 2001-02.

The procedure of this research work has been divided in the following four main categories.

Surface Sterilization of Rice Seeds

Seeds of Basmati-370 and Basmati-385 were provided by rice programmed, Agricultural Biotechnology Institute (ABI), National Agricultural Research Center Islamabad, and it was taken as explant source for callus induction. Healthy and mature seeds were selected by physical appearance and they were dehusked manually. Seeds were first washed with detergent and then rinsed three times with simple tap water. For surface sterilization of seeds Clorox (5.25% sodium hypochlorite) and ethanol was applied. After the applications of Clorox and ethanol seeds were rinsed thrice with autoclaved distilled water. Finally the seeds were dried with autoclaved filter paper, and they were shifted carefully to the culture room of Agricultural Biotechnology Institute.

Basal Media Preparation

M.S and N6 basal media were used for callus initiation. These media were prepared according to the ingredients (Table I). The exact amount of nutrients was dissolved in the distilled water. Two types of growth regulators 2,4-D alone or in combination with BAP was used for callus induction. 2,4-D @ 2.0 and 2.5 mg l^{-1} and BAP @ 0.0, 0.1, 0.5 mg l^{-1} respectively was added in the media. Sucrose at the rate of 3% and agar at the rate of 0.7% was also added in the media. PH of the media was adjusted at 5.78-5.80 with the help of PH-meter. M.S. and N6 media were poured into the test tubes, it was plugged properly and autoclaved at 20 lbs pressure for 15 minutes in the autoclave machine.

Inoculation of Sterilized Seeds

The most important step in tissue culture technique is the inoculation of seeds. This operation was performed in the laminar flow cabinet at the culture room of ABI. Before the operation surface sterilization of the laminar flow unit was carried by UV-light for two minutes. After that hands were disinfected with 75% ethanol to prevent chance of contamination. Dried seeds were then inoculated into test tubes under aseptic condition in laminar flow unit. To minimize chance of infection the instruments were dipped in disinfectant after every operation.

Growth Chamber

Inoculated cultures were incubated at 25±3º C under the influence of 2000-lux light intensity for 16 hours photoperiod. Callus induction of rice seeds were observed after 21- days. The data for callus induction frequency were recorded for two rice varieties on two different media with different concentrations of growth regulators.

Table I ***Composition of MS and N6 media***

Macronutrients		
KNO_3	1900	2830
NH_4NO_3	1650	0
$CaCl_2.2H_2O$	440	166
$MgSO_4$	370	90.37
KH_2PO_4	170	400
$(NH_4)_2\ SO_4$	--	463
Micronutrients		
$MnSO_4.H_2O$	16.9	3.33
H_3BO_3	6.2	1.6
KI	0.83	0.80
$ZnSO_4.4H_2O$	8.6	--
$ZnSO_4.7H_2O$	--	1.5
$NaMoO_4.2H_2O$	0.25	--
$CuSO_4.5H_2O$	0.025	--
$CoCl_2.6H_2O$	0.025	--
Iron source		
$FeSO_4.7H_2O$	27.85	27.85
NaEDTA	37.25	37.25
Vitamins		
Myoinositol	100	--
Nicotinic Acid	0.5	0.5
Pyridoxine HCl	0.5	0.5
Thiamine HCl	0.1	1.0
Glycine	2.0	--
Sucrose	30g 30g	**Agar** 7g 7g

RESULTS AND DISCUSSION

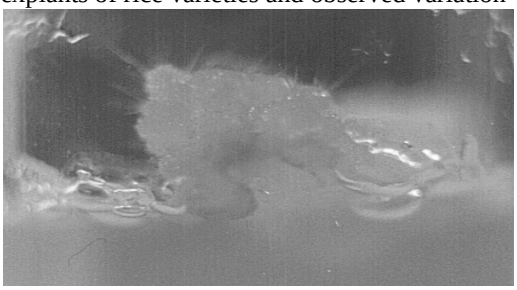
Results obtained from tissue culture techniques of Bas-370 and Bas-385 performed in ABI laboratory and repeated three times from 10th February to

October 2001. Chance of contamination was much low when both the varieties were treated with 50% Clorox and 70% ethanol. Oono (1981) also used seeds as explants source for rice callus induction. The main objective of our study is to know the actual procedure for seed sterilization and callogenesis in these cultivars. The potential of both varieties for callus induction was observed significantly different on M.S and N6 media at different concentrations of growth regulators. It is noteworthy that N6 and M.S media, which proved to be optimum for the growth of callus, were unable to support cell growth in liquid media.

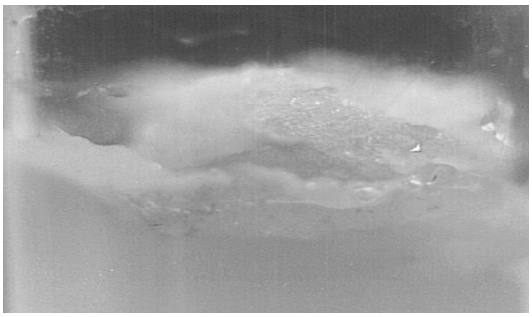
Callus induced from both the varieties were different at various level of 2,4-D and BAP. Maximum callus formation (62.5%) was recorded for Bas-385, followed by Bas-370 (55.55%) when seeds were cultured on MS medium supplemented with 2,4-D@2.0 mgl^{-1} and $2mgl^{-1}$ 2,4-D+0.1mgl^{-1} BAP respectively (Table II and IV). Lowest callus of 41.66 % was observed each for Bas-370 and Bas-385 when MS medium is supplemented with 2.5 and 0.5 mgl^{-1} of 2,4-D and BAP respectively. It is cleared that potential of Bas-385 towards callogenesis was higher than Bas-370. Averaged across MS media callus induction frequency was 48.14 % for BAS-370 vs 53.22 % for BAS-385 (Fig 2). This is equivalent to the net reduction of 5.08 % for BAS-370. Growth regulator 2,4-D at the rate of $2mgl^{-1}$ was suggested and proved to be best for callus induction in both the varieties.

Using N6 media callus induction frequency ranged from 20.83 to 68.05% for both the varieties. When 2,4-D@$2mgl^{-1}$ was supplemented with N6 media, produced highest and good quality callus from Bas-385 (68.05%) and minimum (20.83%) from Bas-370 (Table III and IV). This result indicated positive correlation between 2,4-D and Bas-385 and negative for Bas-370. Average across N6 media callus induction frequency was 23.6 % for Bas-370 vs 60.64 % for Bas-385 (Fig. 3). This is equivalent to the net reduction of 7.42 % for BAS-370. Calli obtained from both the varieties were friable, granular, and yellow in color. So it was proved that N6 media is better for callus induction of rice as compared to M.S medium. Overall result of BAS-385 was best on various concentrations of 2,4-D and BAP on both, MS and N6 media. Callus form Bas-385 was healthy and more fleshy as compare to Bas-370 (Figure 1). The active division of cells was more prominent in the callus of Bas-385, that's why it looks bulky. Secondly Bas-385 had more capacity for producing callus, especially when N6 media supplemented with 2,4-D@$2mgl^{-1}$. Agronomic features of Bas-385 are also better than Bas-370 because it is developed through hybridization between Bas-370 and TN-1. Callus induction must be dependent on the genetic potentials of the variety and secondly the combination of hormones at different concentration.

Callus induction is the best way to create somaclonal variations in crop plants. Variations are the basis for improvement and some time this variation is heritable. Oono (1981) cultured seed explants of rice varieties and observed variation



(a) Calli Obtained form Bas-370



(b) Calli obtained from Bas-385

for certain agronomic characters that are also heritable. Once cultured techniques were established in rice, it become possible to apply them for callus regeneration and transformation.

Table II ***Percentage callus induction frequency and contamination in Bas-370 on M.S Medium***

Treatments	Sterilizing agents		Clorox time/Ethanol time (min)	Hormone in mg L^{-1}		# Of inoculated test tubes	% Contami-nation	% Callus induction frequency
	% Clorox	% Ethanol		2,4-D	BAP			
1	50	70	20/1.0	2.0	0.0	72	58.33%	20.83%
2	50	70	20/2.0	2.0	0.1	72	73.61%	22.22%
3	50	70	20/4.0	2.5	0.5	72	52.77%	**27.77%**

Table III ***Percentage callus induction frequency and contamination in Bas-370 on N6 Medium.***

Treatments	Sterilizing agents		Clorox time/Ethanol time (min)	Hormone used in mg L^{-1}		# Of inoculated test tubes	% Contami nation	% Callus induction frequency
	% Clorox	% Ethanol		2,4-D	BAP			% Clorox
1	50	70	20/1.0	2.0	0.0	72	1	50
2	50	70	20/2.0	2.0	0.1	72	2	50
3	50	70	20/4.0	2.5	0.5	72	3	50

Table IV ***Percentage callus induction frequency and contamination in Bas-385 on M.S Medium.***

Treatments	Sterilizing agents		Clorox /Ethanol time (min)	Hormone used in mg L^{-1}		# Of inoculated test tubes	% Contami nation	% Callus induction
	% Clorox	% Ethanol		2,4-D	BAP			% Clorox
1	50	70	20/1.0	2.0	0.0	72	1	50
2	50	70	20/2.0	2.0	0.1	72	2	50
3	50	70	20/4.0	2.5	0.5	72	3	50

Table V. Percentage callus induction frequency and contamination in Bas-385 on N6 Medium

Treatments	Sterilizing agents		Clorox/Ethanol time (min)	Hormone used in mg L^{-1}		# Of inoculated test tubes	% Contami nation	% Callus induction frequency
	% Clorox	% Ethanol		2,4-D	BAP			% Clorox
1	50	70	20/1.0	2.0	0.0	72	1	50
2	50	70	20/2.0	2.0	0.1	72	2	50
3	50	70	20/4.0	2.5	0.5	72	3	50

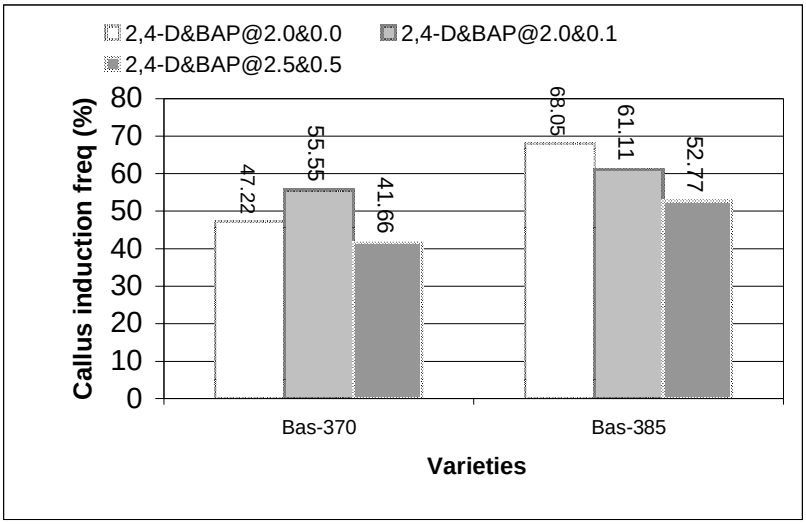


Fig. 2. Callus induction frequency (%) of Bas-370 and Bas-385 on M.S media at different level of 2,4-D and BAP.

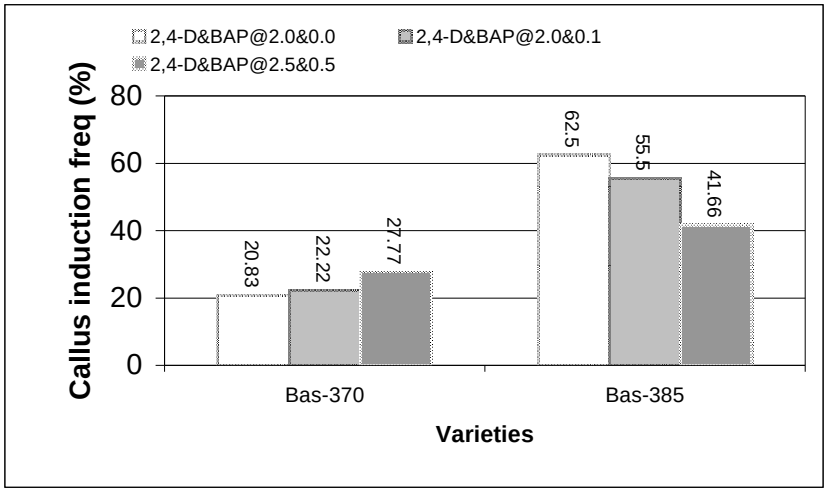


Fig. 3. Callus induction frequency (%) of Bas-370 and Bas-385 on N6 media at different level of 2,4-D and BAP.

CONCLUSION AND RECOMMENDATION

Infections of the callus is main problem in tissue culture technique so we should adopt the following precautions.

1. We should sterilize the seeds in 50% hypochlorite solution for 20 minutes and all the apparatus like flasks, petri plates, blades and forceps etc. should be disinfectant with 70% ethanol. Media should be prepared accurately with respect to concentrations and PH of the media must be maintained properly. Exact amount of growth hormones must be added.

2. Seeds of Bas-370 on MS media with combination 2.0 and 0.1mgl^{-1} should be used to get maximum callus, while hormonal combination 2,4-D and BAP @2.0 and 0.0 mg l^{-1} should be used for better callus induction of Bas-385 on N6 media. Bas-385 is an improved version of Bas-370 because it is developed through hybridization between Bas-370 and TN-1. That's why it is more responsive towards callus as will as high doses of fertilizers. Over all results indicated that Bas-385 having more potentials and suitable for further genetic studies.

REFERENCES

FAO. 2002 Food Outlook Bullet. No: 1,FAO, Rome.

Gonalz, M.C. 2000. Effects of different growth regulators on in vitro culture of rice cultivors. Tropicales. 21(1): 27-28.

Khan, J.A., F.F. Jamil and M.A. Gill. 2000. Screnning of rice varieties against bakanae and bacterial leaf blight. Pak. J. Phytopath. 12(1): 6-11.

Larkin, P.J. and W.R. Scowcraft. 1981. Somaclonal variation a novel source of variability from cell cultures of plant improvement.Theor. Appld. Genet. 60:197-214.

Marasi, M.A., O.A. Bovo, A. Socchi and L.A. Mrongiski. 1996. Cytokining in the callus induction medium for plant regeneration of rice. Phyton Int, J. Exp. Bot. 59(1-2): 155-160.

Murashige, T. and F. Skoog. 1962. Arevised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 473-497.

Navraj, K., M.S. Gill, G. Raman, T.S. Bharaj, S.S. Gosal, N. Kaur and R. Gill. 1999. Factors enhancing somatic embryogenesis and high frequency plant regeneration in rice. Crop Improv. 26(1): 23-27.

Nitsch, J.P. and C. Nitsch. 1969. Haploid plants from pollen grains. Sci. 163: 85-87.

Oono, K. 1981. In vitro methods applied to rice in plant tissue culture. T. Athorpe, ed. Acad. Press, New York. pp. 273-298.

Rashid, H., A. Toriyama, K. Qurashi, Hinta and K.A. Malik. 2000. An improved method for shoot regeneration from calli of Indica rice. Pak. J. Biol. Sci. 3 (12): 2229-2231.

Valdez, M., M. Monoz, J.R. Vega and A.M. Espinoza. 1997. Plant regeneration of Indica rice from mature embryo derived calli. Revistade Biologia Tropical. 44(3): 13-21.

Xie, J.H., M.W. Gao, Q.H. Cai, X.Y. Chens, Y.W. Shen and Z.Q. Liang. 1995 Optimized growth regulators combination in Japonica rice. Plant Cell Tissue and Organ Culture. 42(3): 245-250.

Yamada, Y., T.Q. Yang and D.T. Tang. 1986. Plant regeneration from protoplast derived callus of rice *(Oryza sativa* L.). Plant Cell Reports. 4: 85-88.