ESTABLISHMENT OF CALLUS CULTURE IN Bg-94-1: A COMMERCIALY CULTIVATED RICE IN SRI LANKA**DARSHANIE P. RAJAPAKSE, M.H. MENDIS AND S.D.G. JAYAWARDENE***Plant Genetic Resources Centre, Peradeniya, Sri Lanka.**(Date of receipt : 29 May 1991)**(Date of acceptance : 15 October 1991)*

Abstract: Basic salts were tested to establish a suitable culture medium for callus initiation from mature seeds of Indica rice (cv. Bg 94-1).^{12,11,7,4} All media were supplemented with sucrose (3% w/v), yeast extract (3 g/l) and 2, 4-dichlorophenoxyacetic acid (3 mg/l). Callus initiation occurred in the area comprising the scutellum layer of the seed. Callus formation was better in LS than all other tested media. MS and Y3 media mostly induced coleoptile growth from the seed. Callus initiation and growth was enhanced when high level (9 mg/l) of 2, 4-dichlorophenoxyacetic acid was incorporated in to LS medium. However, subsequent growth of callus was impaired in this medium. Low level (2 mg/l) of 2, 4-dichlorophenoxyacetic acid in combination with 6-benzylaminopurine (0.2 mg/l) is selected for callus establishment from mature Indica seeds.

1. Introduction

Callus culture is a valuable tool in crop improvement programs. It is being applied in creating genetic variability in plants (somaclonal variation) and screening of plants for stress tolerance. Mature seeds are an excellent source for the establishment of embryogenic callus (*E. callus*) in rice since the tissues originating from scutellum and the shoot apical meristem produce embryoids² and seeds can withstand vigorous surface sterilization. Auxin is critical in callus initiation¹³ and 2,4-dichlorophenoxyacetic acid (2,4-D) is considered as the best source of auxin for callus induction in rice.⁶ N6 and MS media have been used for anther and pollen culture of rice.⁵ LS medium has been reported¹⁵ for induction of callus from roots, embryos and leaf bases of the plant.

It is generally accepted that thiamin is a vital component of tissue culture media and other vitamins like nicotinic acid, pyridoxin, biotin and Ca-D pantothenate may be beneficial. Yeast extract (YE) is often included in the culture media for establishment of rice tissue cultures. It causes mitosis in quiescent cells and promotes exuberant growth of callus.¹⁸ Studies have been conducted to observe the effect of light, temperature and pH of culture medium on tissue cultures of Japonica rice.¹⁷ Indica rice is generally recalcitrant to callusing *in vitro*.³ So far only a few cultivars and varieties of Indica rice have been regenerated from tissue cultures. The present study evaluated the effect of different media on tissue culture of Bg 94-1, a commercially grown rice variety in Sri Lanka.

2. Materials and Methods

Dry mature rice seeds (cv. Bg 94-1) were dehusked and surface - sterilized by immersing in 70% ethanol (1 minute) followed by 0.1% HgCl₂ (3 minutes) and washing (3-4 times) with sterilized distilled water.

Media were prepared using MS, LS, Y3 and N6 basic inorganic salts (Table 1). All media were fortified with thiamin hydrochloride (1.0 mg/l) and except for LS medium, with nicotinic acid (0.5 mg/l) and pyridoxin hydrochloride (0.5 mg/l). Sucrose (3% w/v), YE (3.0 g/l) and 2,4-D (3.0 mg/l) were added to each medium. The pH of media was adjusted to 5.7 before dissolving agar (0.8% w/v; Difco-bacto). Each medium was dispensed (10 ml aliquotes) into test tubes or petri dishes and sterilized by autoclaving for 15 minutes at 121°C with steam pressure (1.2 kg/cm²). Sterilized seeds (10 seeds/vessel) were inoculated onto prepared media and cultures were maintained at 26 ± 1°C under fluorescent light (200 - 250 lux; 16 hours) for callus induction.

Table 1: Major and minor inorganic salts and organic components of the four basic media in the study.

Compound	Final Concentration (mg/l)			
	LS	MS	N6	Y3
Major Salts				
(NH ₄) ₂ SO ₄	-	-	463	-
NH ₄ NO ₃	1650	1650	-	-
NH ₄ Cl	-	-	-	535
MgSO ₄ · 7H ₂ O	370	370	185	247
KNO ₃	1900	1900	2830	2020
KCl	-	-	-	1492
CaCl ₂ · 2H ₂ O	440	440	166	284
KH ₂ PO ₄	170	170	400	-
NaH ₂ PO ₄ · 2H ₂ O	-	-	-	312
Minor Salts				
MnSO ₄ · 4H ₂ O	22.3	22.3	4.4	11.2
ZnSO ₄ · 7H ₂ O	8.6	8.6	1.5	7.2
CuSO ₄ · 7H ₂ O	0.025	0.025	-	0.16
KI	0.83	0.83	0.83	8.3
H ₃ BO ₃	6.2	6.2	1.6	3.1
Na ₂ MoO ₄ · 2H ₂ O	0.25	0.25	-	0.24
NiCl ₂ · 2H ₂ O	-	-	-	0.24
Na ₂ EDTA	37.25	37.25	37.25	149.0
FeSO ₄ · 7H ₂ O	27.85	27.85	27.85	111.4
Organic Components				
Thiamin hydrochloride	1.0	1.0	1.0	1.0
Nicotinic acid	-	0.5	0.5	0.5
Pyridoxin hydrochloride	-	0.5	0.5	0.5

LS medium prepared as previously was again tested with 2,4-D at six levels with one treatment in combination with 6-benzylaminopurine (BAP) for further callus establishment (Table 2).

Seed callus induction and growth was examined weekly. Four weeks after seed inoculation, callus initiation efficiency (percentage of inoculated seeds in the vessel which give rise to callus) and wet weight (mg/seed) of calli were recorded (figure 1 and figure 2).

Four weeks old calli (2-5 mm diameter) were plated in MS medium with sucrose (4% w/v), BAP (3.0 mg/l), Indole-3-butyric acid (IBA, 5.0 mg/l) and incubated for 3-4 weeks under 2000 lux at $26 \pm 1^{\circ}\text{C}$ for regeneration of shoots. These shoots (20-40 shoots) with few initiated roots were transferred onto paper bridges in MS medium with 1-naphthaleneacetic acid (NAA, 1.0 mg/l) for 2 weeks to improve root growth. The plantlets formed were then cultured in hormone-free MS solid medium (50 ml in glass jars) for 1-2 weeks to induce better plant growth. These plantlets were transferred to pots containing sterilized soil and maintained in a glass house till maturity.

3. Results

The efficiency of callus initiation and growth in response to different culture media are given in figure 1 and 2 respectively. Callus initiation was observed to occur from the tissue between the growing coleoptile and the endosperm (area comprising the scutellum). The callus initiation efficiency was highest (35%) in LS medium. N6, MS and Y3 media induced the formation of callus but latter two media mostly promoted coleoptile growth.

The highest wet weight (75 mg/seed) of callus was recorded with LS medium. Calli formed in this medium were large and shiny with numerous globular structures on the surface and colour varied from white to light yellow. Formation of such calli was observed to be higher in N6 than in MS medium. Calli formed in Y3 medium were friable and off-white in appearance. Callus induction efficiency was very low with this medium.

Growth response of rice seeds cultured in LS medium containing different levels of 2,4-D (in the presence or absence of BAP) is summarized in Table 2. Callus initiation was rapid at high concentrations (5 and 9 mg/l) of 2,4-D. However, low concentrations of 2,4-D in the presence of BAP also favoured callus growth. These calli remained fresh for a longer period than those formed on media containing higher level of 2,4-D.

Figure 1: Callus initiation efficiency of rice seeds after a culture period of 4 weeks in LS, MS, N₆ and Y₃ media. Each media formulation was replicated five times.

Figure 2: Growth of callus from rice seeds within a culture period of 4 weeks in LS, MS, N₆ and Y₃ media. Each media formulation was replicated five times.

Table 2: Growth response in LS medium containing different levels of 2,4-D (in the presence or absence of BAP) after 4 weeks of seed inoculation.

2,4-D (mg/l)	BAP (mg/l)	Growth response	
		Callus initiation efficiency (%)	Appearance of callus
0.5	-	00	All seeds produced complete plants.
1.0	-	08	Abnormal enlargement of coleoptile. Calli formed degenerated within few days.
2.0	-	22	White, soft calli with root-like appendages.
2.0	0.2	64	White or light-yellow, large, soft, shiny callus (E. callus).
3.0	-	36	White or light-yellow soft callus (E. callus).
5.0	-	40	White or light-yellow shiny compact callus.
9.0	-	66	Light yellow, large, shiny callus; callus diameter < 1.0 cm. Calli formed degenerated in few days.

All observations are based on 5 replicates per treatment.

Callus growth continued for 2-3 weeks in the regeneration medium and subsequently green spots appeared from white, globular areas of the callus. About 10 days later shoots emerged (2-4 shoots per callus) from these green spots. These shoots produced many number of roots within 2 weeks on transfer to rooting medium. The regenerated plantlets grew well upon transfer to soil.

4. Discussion

A major factor which plays a vital role in successful callus culture is the composition of culture medium in which cultures are grown.

Earlier studies reveal that low nitrogen in growth medium favours callus formation in rice.⁸ Results of the present study support this observations since MS medium with 60 mM of total inorganic nitrogen gave lower callus weight than N6 and Y3 media which contained 35 mM and 30 mM total inorganic nitrogen respectively. In contrast, LS medium having same constituents and concentrations of inorganic salts

Figure 3 : Stages of callus culture in rice (cv. Bg 94-1).

- 3.1 Formation of E and NE (non embryogenic) callus from cultured mature seeds.
- 3.2 Regeneration of shoots from callus with few initiated roots.
- 3.3 Plantlet formation from callus on paper-bridge.
- 3.4 Regenerated rice plant ready for pot-transfer.

as MS medium, gave the best performance in callus formation. Color and appearance of calli formed in this medium indicated the formation of E. callus.¹⁴

This may be due to the absence of nicotinic acid and pyridoxin HCl in LS medium. These two compounds promote plant formation than callus from rice embryos. Previous experiments¹⁶ have suggested that, elimination of micronutrients from culture medium is favourable for callus induction from rice anthers. Present study reveals that it is same with rice embryos also, since callus formation was observed in N6 medium which contained micronutrients at low concentrations when compared to LS and MS media. Furthermore, some micronutrients present in LS and MS media are totally absent in N6 medium.

Growth response of cultured rice seeds in LS, MS, Y3 and N6 media do not identify the effect of type and concentration of individual micronutrients on initiation and growth of callus. Therefore, in order to elucidate the relationship between various inorganic components of a culture medium and callus establishment, further investigations are necessary.

Higher concentrations of 2,4-D enhance callus induction. However, this study indicated that calli formed at high level of 2,4-D (5.0 > mg/l) degenerate soon when transferred to regeneration medium. It has been discussed that high concentration of 2,4-D inhibits regeneration from callus by affecting shoot and root formation (morphogenesis) from cultured tissues.¹⁰ Furthermore, in combination with a cytokinin such as kinetin and BAP, 2,4-D plays a beneficial role in rice tissue culture as shown by several workers.^{1,9}

Acknowledgements

The authors wish to thank Prof. M.D. Dassanayake for the guidance given in preparation of this article, Mr P.K. Samarajeewa and Mr S. Balendira for the help given, Mr K. Gunatunga for photography and Ms. C.K. Ratnayake for typing the manuscript.

References

1. ABE, T. & FUTSUHARA, Y. (1986) *Japan J. Breed* 36: 1-6.
2. ABDULLAH, R. (1987) The development of a reproducible system for efficient plant regeneration from rice (*Oryza sativa* L.) protoplasts. Ph.D. thesis, University of Nottingham, U.K., pp. 42-43.
3. CHALEFF, R.S. (1979) In Innovative approaches to rice breeding, IRRI, Los Banos, pp. 88-91.
4. CHU, C.C., WANG, C.C., SUN, C.S., HSU, C., YIN, K.C., CHU, C.Y. & BI, F.Y. (1975) *Scientia Sinica*. 18: 659-668.
5. CHU, C.C. (1978) In Proceedings of Symposium of Plant Tissue Culture, Peking, 25-30 May 1978, pp. 43-50, Science Press.

6. DYKES, T.A. & NABORS, M.W. (1986) In Rice Genetics, Proceedings of the International Rice Genetics Symposium, IRRI, 27-31 May 1985, pp 799, Philippines.
7. EEWENS, C.J. (1976) *Physiol. Plant.* 36: 23-28.
8. GUPTA, H.S. & BORTHAKUR, D.N. (1987) *Theor. Appl. Genet.* 74: 95-99.
9. HEYSER, J.W., DYKES, T.A., DEMOTT, K.J. & NABORS, M.W. (1983) *Plant Sci. Lett.* 29: 175-182.
10. HUANG, C.R., WU, Y.H. & CHEN, C.C. (1985) In Rice Genetics, Proceedings of the International Rice Genetics Symposium, IRRI, 27-31 May 1985, pp. 769-770.
11. LINSMAIER, E.M. & SKOOG, F. (1965) *Physiol. Plant.* 8: 100-127.
12. MURASHIGE, T. & SKOOG, F. (1962) *Physiol. Plant.* 15: 473-497.
13. MURASHIGE, T. (1974) *Ann. Rev. Plant Physiol.* 25: 135-166.
14. NABORS, M.W. & DYKES, T.A. (1985) In Biotechnology in International Agricultural Research. Proceedings of the Inter Centre Seminar on IARCS IRRI. pp. 121-138 IRRI, Manila.
15. NISHI, T., YAMADA, Y. & TAKAHASHI, E. (1968) *Nature* 219: 508-509.
16. SONG, H.G., LU, S.N., LI, G.R., YUN, S.G. & LI, J.W. (1978) In Proc. Symp. Plant Tissue Culture, pp. 97-106, Peking, Science Press.
17. YAMADA, Y. & LOH, W.H. (1984) In Ammirato, P.V., EVANS, W.R., SHARP, W.R. & YAMADA, Y., Hand Book of Plant Cell Culture, Vol.3, pp. 151-169, New York, McMillan Publishers.
18. YATAZAWA, M., FURUHASHI, K. & SUZUKI, T. (1967) *Plant Cell Physiol.* 8: 363-373.