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ESTABLISHMENT OF REGENERATING CALLI AND CELL SUSPENSION LINE OF BASMATI RICE (*ORYZA SATIVA* L. CV. B.370)

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Abstract

Methods were developed for callus induction and efficient plant regeneration from mature embryos and immature inflorescences of basmati rice (*Oryza sativa* L. cv. Basmati-370). Callusing from seeds of rice was observed in MS or N6 media with various levels of 2,4-D. Somatic embryogenesis was achieved in both media however, response was better in the presence of Kinetin (2 mg l^{-1}). Using field grown immature inflorescences and embryos induction of callus and regeneration was observed. Cell suspension cultures were initiated from 2-3 months old calli of B-370. A fast growing and finely dispersed cell line of Basmati-370 was established. Regeneration of plants was achieved in MS-0 (no hormone).

Introduction

The potential for genetic improvement of monocots, using *in-vitro* techniques including somatic hybridization has recently been applied in rice (Kyozuka *et al.*, 1987; Tereda *et al.*, 1987). *Oryza sativa*, 'indica'exclusively grown in Pakistan occupies 80% of the cultivated rice in the world (Kyozuka *et al.*, 1988). Most of the studies reported earlier deal with the 'japonica' sub- type (Abdullah *et al.*, 1986; Yamada *et al.*, 1986; Kyozuka *et al.*, 1987). Recent interest in 'indica' type of rice resulted in several reports of successfully regenerating plants from protoplasts obtained from cell suspensions of 'indica' sub species (Kyozuka *et al.*,1988; Lee *et al.*, 1989; Wang *et al.*, 1989; Datta *et al.*, 1990;). In addition to standard breeding programmes, there is need to develop reproducible techniques of regenerating protoplasts of indica rice for employing somatic fusion and other transformation techniques for obtaining stress tolerant crop.

In Pakistan, a preliminary plant regeneration procedure through callus has been reported for some local cultivars including B-370 (Abbas *et al.*, 1985). The present paper reports for the first time on procedures for high efficiency *in-vitro* plant regeneration and development of fast growing cell suspension of basmati rice.

Materials and Methods

Plant material: Seeds of basmati rice (*Oryza sativa* L. cv. B-370) were obtained from the Mutation Breeding Division of NIAB, Faisalabad. Rice seeds dehusked manually were washed with detergent and surface sterilized in 20% (v/v) NaOCl for 30 minutes with constant shaking followed by washing in sterilized distilled water. Embryos were excised from the seeds and 10-15 embryos were placed in jars containing 50 ml of callus initiation media. For aseptically grown rice seedlings, seeds were surface sterilized as described

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Table 1. Effect of different media on callus initiation of basmati rice (*Oryza sativa* cv. B. 370) from 3 explants.

Explant/hormone-media* (2,4-D)		No. of seeds	Callus initiation	% Callus initiation**
Mature seeds/				
0.5	MS	67	19	28
	N6	15	5	33
1.0	MS	67	34	60
	N6	15	6	40
2.0	MS	54	46	85
	N6	30	21	70
4.0	MS	31	15	48
	N6	22	8	36
Immature embryos/ 2.0+				
0.2 mg l^{-1} BAP	MS-3	45	20	44
2.0	MS	14	10	71
Immature Inflorescences/				
	MS-3	59 (1-2 mm cut portions)	30	51

*2,4-D in mgl^{-1}. For media composition see materials and methods.

**Callus initiation response in % is presented in round figures.

above and germinated aseptically on hormone free (MS-0) medium. The roots of 6-8 day old seedlings were cut into small pieces and used as explant for developing cell suspension. Immature embryos and immature inflorescences were collected from field during Sept-Oct., 1989 and 1990.

Callus Induction and Regeneration: Surface sterilized, excised embryos of rice were placed on MS and N6 media containing 3% sucrose and 0.5, 1, 2 and 4 mg l^{-1} 2,4-D. Cultures were incubated in continuous light (2000 lux) at 24±2°C. After 30 days, a part of developing calli were placed on regeneration media containing different concentrations of IAA, NAA, BA and kinetin. The remaining callus was transferred to maintenance media with 1 mg l^{-1}2,4-D. Developing somatic embryos were recorded when the shoots were at least 1 cm high.

Suspension Culture: Suspension cultures of Basmati rice were initiated by transferring 200-400 mg of friable callus (ca. 2 months old) into 125 ml Erlenmyer flasks containing 30 ml of (AA) amino acid (Toriyama & Hinata, 1985), B5 (Gamborg *et al.*, 1968), N6 (Chu *et al.*, 1975) and MS (Murashige & Skoog, 1962) liquid media with 2 mg l^{-1} 2,4- D. The flasks were placed on a gyratory shaker at 120 rpm in dark. The cell cultures were maintained by removing used media and adding fresh media after 3-4 days. Microscopic examinations of cell cultures was made periodically on inverted microscope (Nikon). Fast growing cultures were filtered through 760 μm and 450 μm sieves, and the cells collected below 450 μm were further cultured on AA media with 2 mg l^{-1} 2,4-D. Sub culturing was performed at intervals of 7-10 days using 1:4 inoculum/fresh medium dilution ratio. Growth was monitored by determining the fresh and dry weights and packed cell volume (PCV) of five replicate samples.

Table 2. Effect of different media on regeneration of basmati rice.

Explant Source	Media*	Calli (No.)	Green plants (No.)	Green plants /callus (No.)	Albino plants (No.)	Albino plants /callus (No.)
Mature seed	MS-R	10	6	0.6	---	---
	N6-RK2	24	18	0.75	10	0.20
	N6-RK5	10	---	---	---	---
	N6-RB2	15	---	---	---	---
Immature embryo	MS-R	20	---	---	---	---
	N6-RK2	10	---	---	---	---
	N6-RK5	15	---	---	---	---
	N6-RB2	10	---	---	---	---
Immature Inflore-scences	MS-R	5	15	3	---	---

*MS-R contains BAP 0.5 mg l^{-1} and NAA 0.05 mg l^{-1}: N6- RK2 contains kinetin 2 mgl^{-1}:N6-RK5 contains kinetin 5 mg l^{-1} and N6-RB2 contains BAP 2 mgl^{-1}.

For plants regeneration, cell clumps below and above 760 m fractions were transferred to Petri dishes or jars containing MS, B5 or N6 agar media with or without plant growth regulators. Suspensions were placed on regeneration media after establishing fast growing cell lines (ca. 6 months).

Results and Discussion

Callusing and Regeneration: Callus formation was noticed in mature seeds of Basmati rice within 2-3 weeks. The calli produced on MS and N6 media were light yellow, compact and granular in structure (Fig 1). The callusing response was more on MS than on N6 medium (Table 1). 2,4-D @2 mg l^{-1} induced more callus in both the media . In addition to genotypic effect (Mikami & Kinoshita, 1988), media composition has also been reported to influence callus induction/regeneration of rice (Peterson & Smith, 1991). For regeneration studies the calli were pooled together irrespective of the basal media used.Of the various media, having variable concentrations of hormones the best response was obtained in N6 medium with 2 mg l^{-1} Kinetin (N6-RK2) (Table 2). Albino plants (0.20 plants/callus) were also obtained. In an earlier report of Raina *et al.*, (1987), a high rate of albino plants (upto 30%) were observed in B-370. Zimny & Lorz (1986) reported that use of 7 mg l^{-1} Dicamba (auxin) resulted in the development of only albino plants in japonica rice (Taipei-309) while at lower concentrations of dicamba or other auxins normal green plants were obtained. It may possibly be due to chromosomal

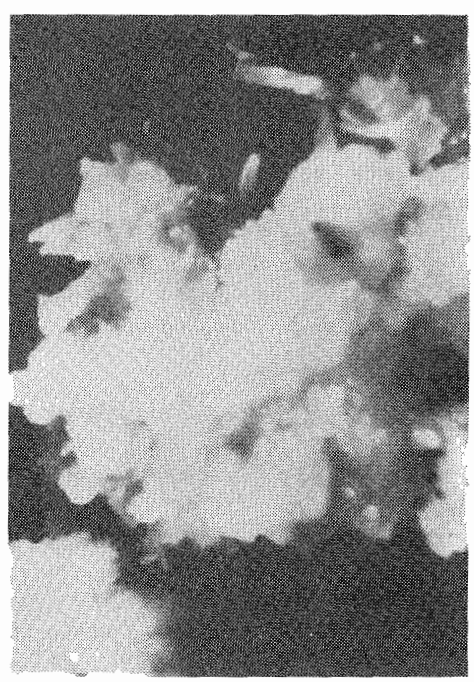

Fig.1. Callus formation from mature seeds of basmati rice (*Oryza sativa* L var B.370) after 6 weeks in culture (x30).

Table 2. Effect of different media on regeneration of basmati rice.

Explant Source	Media*	Calli (No.)	Green plants (No.)	Green plants /callus (No.)	Albino plants (No.)	Albino plants /callus (No.)
Mature seed	MS-R	10	6	0.6	---	---
	N6-RK2	24	18	0.75	10	0.20
	N6-RK5	10	---	---	---	---
	N6-RB2	15	---	---	---	---
Immature embryo	MS-R	20	---	---	---	---
	N6-RK2	10	---	---	---	---
	N6-RK5	15	---	---	---	---
	N6-RB2	10	---	---	---	---
Immature Inflore-scences	MS-R	5	15	3	---	---

*MS-R contains BAP 0.5 mg l^{-1} and NAA 0.05 mg l^{-1}; N6- RK2 contains kinetin 2 mgl^{-1}:N6-RK5 contains kinetin 5 mg l^{-1} and N6-RB2 contains BAP 2 mgl^{-1}.

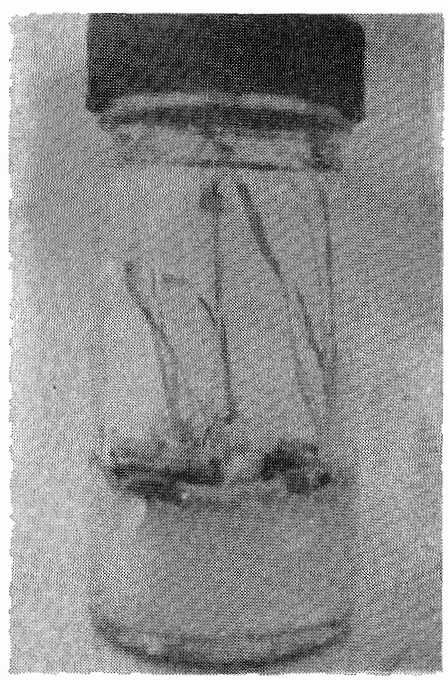

Fig.2. Plant regeneration of rice cv. Basmati-370.

abberrations during rapid growth of plant cells under the influence of plant growth hormones. In our later studies (data not shown), no difference in regeneration was observed using N6 or MS media. It would therefore, suggest that kinetin was responsible for higher regeneration rate in N6 media.

Immature inflorescences (Wang & Zapata, 1987) and immature embryos (Lee *et al.*, 1989) of field grown rice were used as explant source. Since field grown immature tissues showed a persistent bacterial contamination, the tissues were sterilized using ethanol for 1 minute and 50% commercial bleach for 30 minutes. The callusing response of both these plants are given in Table 1. The calli obtained from immature inflorescences on MS-3 medium (Wang & Zapata, 1987) were light yellow to white, compact and granular. For regeneration studies, MS media was employed. The frequency of plant formation from the calli of immature inflorescences was 3.0 plants/callus as compared to 0.75 plants/callus from mature seeds and nil in immature embryos. Immature embryos showed upto 71% callusing response on MS medium with 2 mg l^{-1} 2,4-D as compared to 44% in MS-3 medium. The use of immature embryo for the production of embryogenic calli and as a source material for initiation of cell suspension lines have been recommended by Lee *et al.*, (1989). In the present study, the timing of collection of immature embryo was not standardized. Thus, the high embryogenic potential could not be obtained. Various media based on the formulation of MS and N6 media with varied combinations of growth hormones did not produce *in- vitro* plants from the calli initiated from immature embryos of B- 370.

Suspension Culture: The suspension cultures of 'indica' rice (*Oryza sativa* cv. B.370) were established which were obtained from calli initiated from seeds and root cuttings. The N6 and MS media which proved to be optimum for the growth of callus were unable to support cell growth in liquid media. Use of modified AA medium (Toriyama & Hinata, 1985) with 2 mg l^{-1} 2,4-D resultd in faster cell growth since AA medium is rich in organic nitrogen which may be needed to achieve rapid growth and proliferation of cell clusters. The cells were mechanically separated by passing through 760 μm and 450 μm sieves. The fraction below 760 μm filter showed establishment of growing cell line of B-370.

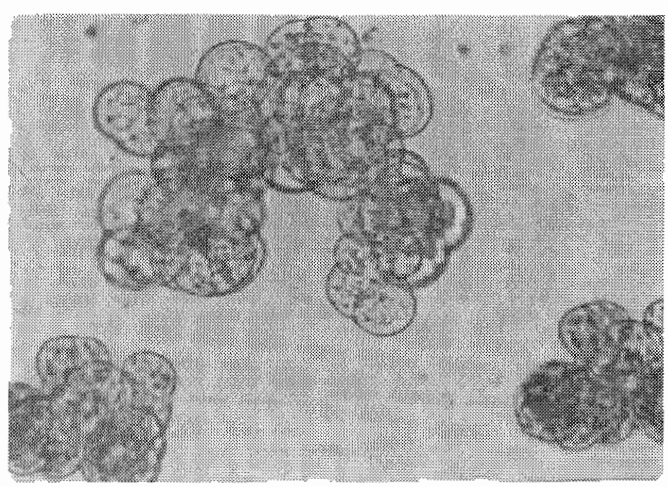

Fig.3. Suspension cultures of B-370-S on AA medium with 2 mg l^{-1} 2.4-D. (x200).

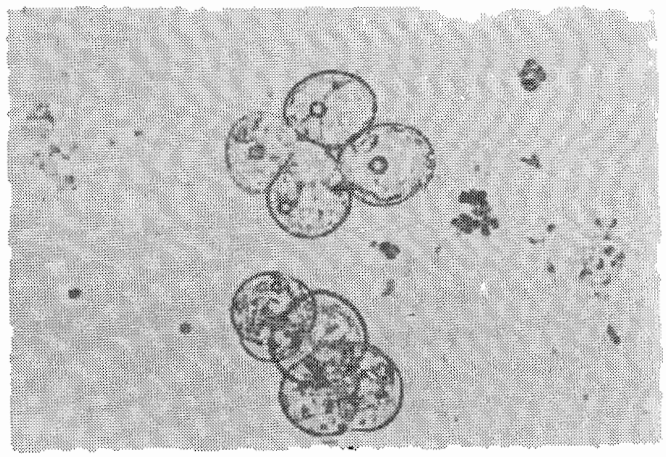

Fig.4. Close-up of isodiametric cells with highly granular cytoplasm of cell suspension of B-370-S (x400).

Microscopic examination of cultures at initial stage revealed the presence of mixture of long vacuolated cells, callus pieces, cell clusters and non dividing cells. The cells of newly established cell lines consisted of isodiametric cells with highly granular cytoplasm which produced small aggregates of various sizes (Figs.3 & 4).

The suspension cultures initiated from shoots, chopped whole seedlings and leaf turned brown after 2-3 weeks which ultimately died. Following the method of Abe & Futsuhara (1986) who used modified MS medium (MS-T) to develop cell line from calli initiated from roots of 'indica' rice 'Chyokota', cell line of B-370 were successfully established.

The data in Fig. 5 shows the growth (2 mg l^{-1} 2-4-D) of cell suspension of B-370-S. The doubling time based on PCV and fresh and dry weights was 4 days. PCV and dry weight co-related well and subsequent studies were then carried out using PCV.

For regeneration studies, cell clumps 760 μm and other fractions (760-120 μm) were placed on MS and N6 media. Three plants were obtained on MS-O medium (no hormone) from cell line of B-370-S. However, in later transfers (at each sub culture) only green spots were observed which were unable to grow into plants. It is known in cereals that the morphogenic capacity of cell suspensions can be partially or completely lost with the culture age (Jones, 1989). Repeated transfer procedures resulted in regeneration of cell suspension of B-370-S into green plants in few jars. Experiments are in progress to refine the method for reliable and routine regeneration of plants. Therefore, methods for the continuous production of regenerable suspensions are needed. The results obtained by Abdullah *et al.*, (1986) for japonica rice (Taipie-309) and Lee *et al.*, (1989) for indica rice (IR- 54), demonstrated that cell suspensions maintained the morphogenic capacity after two years in culture. This points to the major role of genotype in selecting a regenerable cell line.

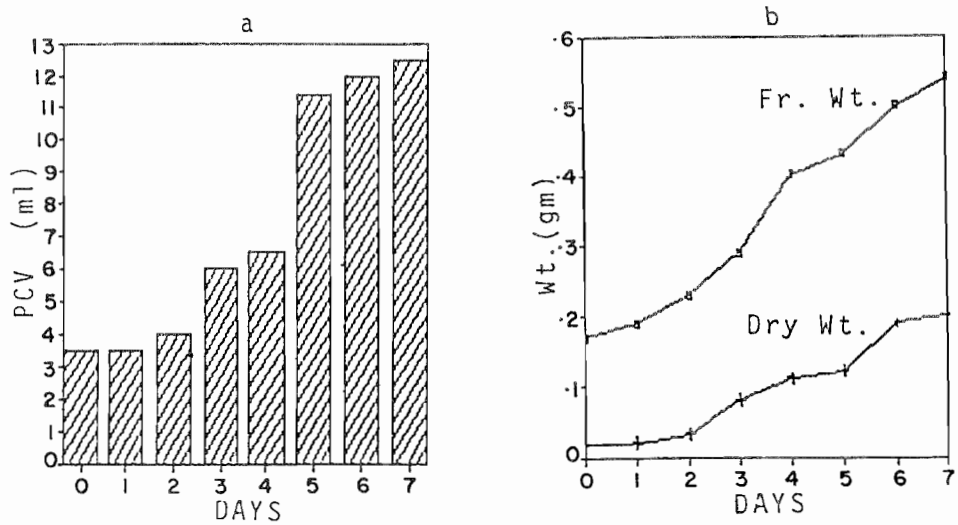

Fig.5. Growth rate of cell suspension of B-370-S on AA medium with 2 mg l^{-1} 2,4-D. Suspensions were grown in 125 ml flasks on a gyrarotary shaker at 120 rpm at 25±2°C. a) Packed cell volume PCV (ml) vs. days. b) Fresh and dry weights (gms) vs. days.

Acknowledgments

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References

Abbas, T. and A. Qureshi. 1985. Studies on callus induction from the anthers of Pakistani rice varieties Bas mati 370, Kashmir Basmati, IR-6 and KS-282. In: *Plant tissue culture* (Ed.) I. Ilali, University of Peshawer, pp 27-32.

Abdullah, R., E.C. Cocking, and J.A Thompson. 1986. Effect of plant regeneration from rice protoplasts through somatic embroygenesis. *Biotechnology*, 4: 1087-1090.

Abe, T. and Y. Futsuhara. 1986. Genotypic variability for callus formation and plant regeneration in rice. *Theor. Appl. Genetics*, 72: 5-10.

Chu, C.C., C.C. Wang, C.S. Sun, C.H. Su. C. Yin, C.Y. Chu, and F.Y. Bi. 1975. Establishment of anefficient medium for anther culture of rice through comparative experiments on the nitrogen sources. *Sci. Sin.*, 18:659-668.

Datta, S.K., K. Datta, and I. Potrykus. 1990. Fertile indica rice plant regenerated from protoplasts isolated from microspore derived cell suspensions. *Plant Cell Reports*, 9: 253-256.

Gamborg, O.L., R.A. Miller, and K. Ojima 1968. Nutrient requirements of suspension cultures of soybean root cells. *Exp. Cell Research*, 50: 151-158.

Jones, M. 1988. Fusing plant protoplasts. *Trends in Biotechnology*, 6: 153-158.

Kavi Kishore, P.B. and G.M. Reddy. 1986. Regeneration of plants from long term cultures of *Oryza sativa* L. *Plant Cell Reports,d, 5: 391-393.*

Kyozuka, J., Y. Hayashi, and K. Shimamoto. 1987. High frequency plant regeneration from rice protoplasts by novel nurse culture methods. *Mol. Gen. Genetics*, 206: 408-413.

Kyozuka, J., Y. Hayashi, and K. Shimamoto. 1988. Plant regeneration from protoplasts of indica rice genotypic differences in culture response. *Theor. Appl. Genetics*, 76: 887-890.

Lee, L., R.E. Schroll, H.D. Grimes, and T.K. Hodges. 1989. Plant regeneration from indica rice (*Oryza sativa* L.). *Planta*, 178: 325-333.

Murashige, T.I. and F. Skoog. 1962. Revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiol. Plantarum*, 15: 473-497.

Peterson, G. and R. Smith. 1991. Effect of abscisic acid and callus size on regeneration of american and inter national rice varieties. *Plant Cell Reports*, 10: 35-38.

Raina, S.K., P. Sathish, and K.S. Sarma. 1987. Plant regeneration from *in vitro* cultures of anthers and mature seeds of rice (*Oryza sativa* L.) cv. Basmati-370. *Plant Cell Reports*, 6: 43-45.

Tereda, R., J. Kyozuka, S. Nishibayashi, and K. Shimamoto. 1987. Plantlet regeneration from somatic hybrids of rice (*Oryza sativa* L.) and barnyard grass (*Echinochloa oryzicola* Vasing). *Mol. Gen. Genetics*, 210: 39-43.

Toriyama, K. and K. Hinata. 1985. Cell suspension and protoplast culture in rice. *Plant Science*, 41: 17-183.

Wang, M. and F. J. Zapata. 1987. Somatic embryogenesis and plant regeneration in tissue culture of *Echino chloa glabrescens* Munro ex Hook F. J. *Plant Physiology*, 130: 79-85.

Wang, D., P.D. Miller, and M.R. Sondahl. 1989. Plant regeneration from protoplasts of indica type rice and CMS rice. *Plant Cell Reports*, 8: 329-332.

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

Zimny, J. and H. Lorz. 1986. Plant regeneration and initiation of cell suspension from root tip derived callus of *Oryza sativa* L. *Plant Cell Reports*, 5: 89-92.

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