Pakistan J. Agric. Res. Vol. 15 No. 1, 1994

ESTABLISHMENT OF CELL SUSPENSION LINES OF INDICA RICE

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ABSTRACT: Cell suspension cultures were initiated from 2-3 months old calli of eight cultivars of indica rice (*Oryza sativa* L.). The calli were originated from mature embryos, immature embryos and immature inflorescences. Seven to ten days old root segments of aseptically grown 8 rice cultivars were also employed to develop cell suspensions. Several liquid media i.e., MS, MS-T and Amino acid (AA) with a range of auxins (0.5 to 8.0 mg/l 2,4-dichlorophenoxy acetic acid) were used. After repeated selection, AA medium with 2.0mg/l 2,4-D was found to be optimum for the growth and maintenance of cell lines. Five fast growing suspension lines were established from 3 cultivars (DM-25; B-370 and B. Kashmir). The cell lines of other cultivars are being developed. Most of the suspensions developed roots on MS or N_6 regeneration media. Sporadic somatic embryogenesis were obtained in two cell lines of B-370 and B. Kashmir. The doubling time of DM-25-Inf and B-370-S in AA medium was found to be 3 and 4 days respectively. Maximum protoplast yield of newly established cell line (DM-25-Inf) was obtained with enzyme mixture of 150mg cellulose R-10, 15mg Pectolyase and 50mg macerozyme in 10 ml CPW at pH 6.0 and 0.6M mannitol as osmoticum.

Key Words: Oryza sativa; Varieties; Cell Lines; Enzymes; Protoplast; Pakistan.

INTRODUCTION

Establishment of a friable cell suspension consisting of small clusters of rapidly dividing cells may be a critical step in the successful isolation, culture and plant regeneration from cereal protoplasts (Jones, 1985). Regeneration of whole plants from cell suspensions and protoplasts have been reported with several genotypes of rice (Abe & Futsuhara, 1986, Abdullah et al., 1986, Toriyama, et al., 1988 and Zimny & Lorz, 1986). The availability of suspension cultures resulted in obtaining protoplasts and subsequent plant regeneration (Fujimura et al., 1985, Thompson et al., 1986, Yamada et al., 1986) and provided materials for transformation studies in rice (Uchimiya et al., 1986, Toriyama et al., 1988, Zhang & Wu, 1988, Shimamoto et al., 1989). Most of the studies reported earlier in the literature deal with the japonica sub-type of *O. sativa* except the report of Abe & Futsuhara (1986) who obtained cell suspension and subsequently plant regeneration from an indica type cultivar, chyokoto. Recent interest in 'indica' rice as it covers more than 80% of the rice growing area of the world resulted in several reports of successfully regenerating plants from protoplasts, obtained from cell suspensions of 'indica' rice varieties (Kyozuka et al., 1988, Lee et al., 1989, Wang et al 1989, Datta et al., 1990, Nayak and Sen, 1990). The present study deals with the development of cell suspension lines of eight cultivars of 'indica' rice for the on-going project on improvement of rice through biotechnological means. Some data regarding isolation and culturing of protoplast is also described.

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MATERIALS AND METHODS

Plant Material

Seeds of 8 cultivars of 'indica' rice were obtained from Mutation Breeding

Division of NIAB, Faisalabad (Table 1). Parts of immature inflorescence and immature embryos were collected from field grown rice during October-November, 1989. For aseptically grown rice seedlings, seeds were surface sterilized using 50% (v/v) sodium hypochlorite for 30 minutes and washed several times with sterilized distilled water and germinated aseptically on hormone-free MS (MSO) medium. Whole seedlings were cut into small pieces and roots of 6-8 day old seedlings were used as explant for developing cell suspension. Two month old calli originated from matures seeds, immature embryos and immature inflorescence were also employed to establish cell lines of 'indica' rice.

Suspension Cultures

Suspension cultures of eight 'indica' rice varieties were initiated by transferring 300-500 mg of friable, creamy, embryogenic callus into 125 ml Erlenmeyer flasks containing 20-30 ml of AA, N6, MS or B5 liquid media with 0.5 mg/l to 8.0 mg/l of 2,4-D. The flasks were placed on a gyratory shaker at 120 rpm under light and/or dark. The cell cultures were maintained by removing used media and adding fresh medium after 4-7 days or earlier if tissues turned brown. Care was taken not to reduce the cell density. Microscopic examination of cell cultures was performed periodically on Nikon inverted microscope. Only fast growing cultures were filtered through 760 uM sieve. The cell aggregates collected below the 760 uM were cultured further on AA or MS media. Repeated sub-cultures at intervals of 7-10 days resulted in the establishment of fast growing cell lines. Cultures were maintained on AA medium with 2mg/L 2,4-D by subculturing at 2

Table 1: Indica rice cultivars and their characteristics

Sr.No.	Name	Characteristics
1	B-370	Fine, long grain world famous established variety.
2	B-Pak	Fine aromatic and long grain basmati variety by a cross C-6222 x BV-370.
3	B-Kashmir	Early maturing mutant of B-370.
4	FG-6	Fine grain mutant of IR-6
5	DM-25	Fine semi dwarf mutant of B-370
6	NR-1	Salt tolerant recombinant of cross Jhona x Magnolia.
7	J-349	Salt tolerant land race variety
8	IR-6	Coarse, standard

week intervals using a 1:4 inoculum/fresh medium dilution ratio. Growth was monitored by determining the packed cell volume (PCV), fresh and dry weights of replicate samples. The root sections of 7-10 days old aseptically grown rice were placed in flasks as described above for callus material. Small calli developed from the cut and wounded ends of the roots. After removing the tissue, the calli were repeatedly sub cultured every 2 weeks on MS-T and AA media with 2mg/L 2,4-D.

Plant Regeneration

For plant regeneration, cell clumps > 760 and 760-120uM fractions were transferred to petri dishes/jars containing MS or N_6 agar media with or without plant growth regulators. Repeated transfers of embryogenic calli developed from cell clumps were also performed in later experiments for achieving plant regeneration.

Protoplast Isolation

Three to four days old suspension cultures of DM-25-Inf were used for the isolation of protoplast. Protoplasts were isolated by incubating nearly 500 mg to 1gm cells in 10ml enzyme solution consisting of salts, MES buffer, enzymes (cellulose Onozuka R-10 150 mg, pectolyase 'Sigma' 15 mg macerozyme 'Yakulut' 50mg) and mannitol 0.6M, and incubated for 6-9 hours at 28 ± 1°C. The mixture was purified by passing through nylon mesh (60 um, 20 um) followed by density gradient centrifugation with 20% sucrose and washing in medium containing 0.6M mannitol as osmoticum. Purified protoplasts were cultured in KMP_8 medium with 1.6% agarose. For culturing protoplasts, feeder-layer plating method (Kyozuka et al. 1987) was also used where a suspension culture of B-370 was used as feeder.

RESULTS AND DISCUSSION

The suspension cultures of eight cultivars of 'indica' rice *(O. sativa)* were established which were originated from calli initiated from seeds, immature embryos and immature inflorescence (Table 2). Suspension cultures were obtained and plants were regenerated from 'japonica' rice (Taipie-309) by Abdullah et al. (1986). They described the use of seedlings, leaf, mature embryo and scutellum derived callus for developing cell suspension cultures of rice. Working with such a strategy, we have also been able to establish suspension lines of 8 cultivars of 'indica' rice. It is noteworthy that N6 and MS media which proved to be optimum for the growth of callus were unable to support cell growth in liquid media. In the absence of any established general technology, a variety of protocols (Abdullah et al., 1986, Zimny & Lorz, 1986, Abe & Futsuhara, 1986 and Lee et al., 1989) were tested. Use of AA medium with 2mg/L 2,4-D resulted in faster cell growth. AA medium is rich in organic nitrogen which may be needed to achieve rapid growth and proliferation of cell clusters. The mechanical separation of cells was made by passing through the 760 um filter. The fractions below 760um filter resulted in establishing rapidly growing cell lines (Table 2). The microscopic examination of the cultures at initial stages revealed the presence of mixture of long vacuolated cells, callus pieces, cell clusters and non-dividing single cells. The

Table 2. Some characteristics of cell suspensions of eight varieties of indica rice (*Oryza sativa*)

Rice culture	Explant	Callusing Media	Cell susp. Media	Regen. Media	Regeneration
B-370	Mature seeds	MS-2 MS-3	AA	MS-D	Sporadic plant formation/roots
				MS-O	Green spots/root
				N6-K2	No response
				N6-K5	No response
	Roots 7-8 day old	MS-T	MS-T AA	MS-D	Green spots/root
				MS-O	Green spots
				N6-K2	Root formation
				N6-K5	Root formation
DM-25	Immature inf.	MS-3	AA	MS-D	Roots
				MS-O	Roots
				MS-18	Proliferating roots
				N6-K2	No response
				N6-K5	No response
	Roots	MS-T	MS-T AA	As above	Roots
B-Kas	Mature seeds	MS-2	AA	"	Green spots/root
	Immature embryo	MS-3	AA	"	No response
	Roots	MS-T	MS-T AA	"	Green spots/root Root formation
J.349	Immature embryo	MS-3	AA	"	No response
	Roots	MS-T	MS-T AA	"	Roots frequent
NR-1	Roots	MS-T	MS-T AA	"	Roots
B-Pak	Roots	MS-T	MS-T AA	"	Roots
FG-6	Roots	MS-T	MS-T	"	Green spots/root
IR-6	Roots	MS-T	MS-T	"	Roots

N6-K2 = N6 medium + 2mg/l Kinetin;N6-K5 = N6 medium + 5mg/l Kinetin; MS-D = MS medium + 0.05 mg/l NAA and 0.5 mg/l BAP; MS-T= MS medium + 2mg/l 2,4-D and 1g/l casein hydrolysate; MS-3= MS medium + 2 mg/l 2,4-D and 0.2 mg/l BAP.

cells of newly established cell lines consisted of isodiametric cells with highly granular cytoplasm and produced small aggregates of various sizes (Figures 1 & 2).

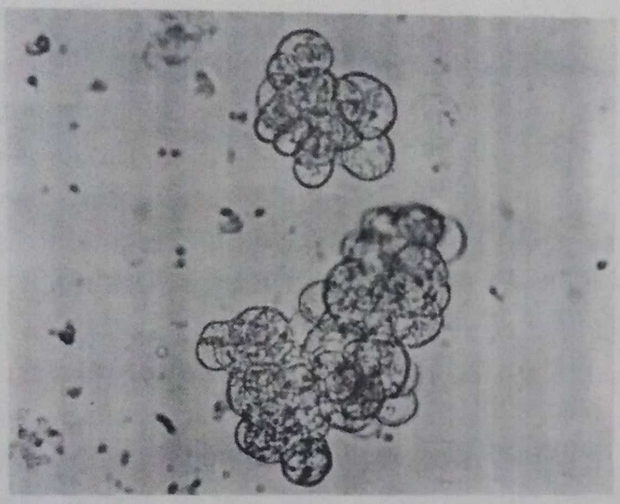

Figure 1. Variation in clump sizes of suspension cultures of B-370-S (X 200).

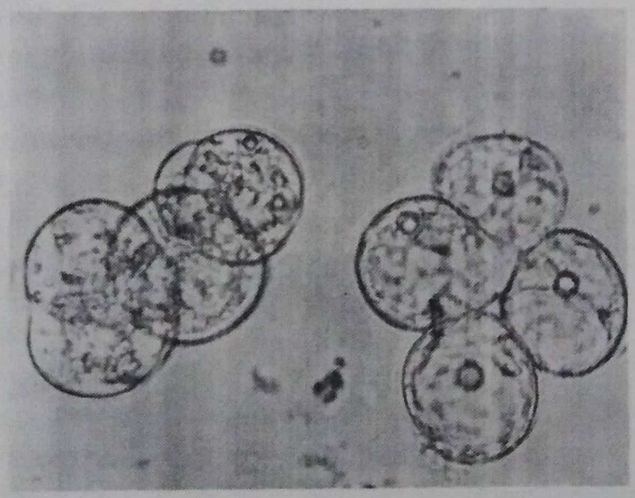

Figure 2. Close-up of isodiametric cells with highly granular cytoplasm of cell suspension of B-370-S (X-400).

The suspension cultures initiated from shoots, chopped whole seedlings and leaf turned brown after 2-3 weeks and 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 'chyokoto' we were successful in establishing cell lines of eight cultivars of rice.

The data in Figures 5 & 6 shows the growth rate over a 7 day period on AA medium (2mg/l 2,4-D) of cell suspensions of DM-25-Inf and B-370-S. The doubling time based on packed cell volume (PCV) and fresh and dry weights were found to be 3 and 4 days respectively. PCV correlated well with weights (fresh & dry) and subsequent studies were done by using PCV.

For regeneration studies, cell clumps >760uM and other fractions (760-120uM) were placed on modified MS or N6 media. The cell aggregates were also placed on these basic media without addition of hormones.

Three plants were obtained in MS-0 medium (no hormone) from cell line of B-370-S which originated from calli of

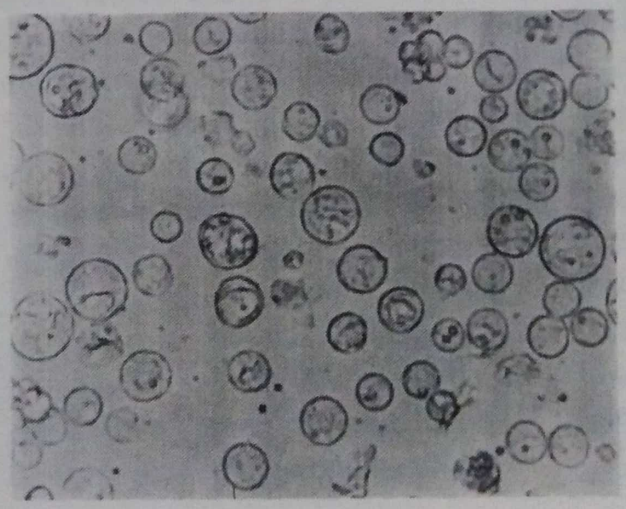

Figure 3. Freshly isolated protoplasts of DM-250-Inf before placing them on sucrose (20%) cushion (X-200).

mature seeds. However, in later transfers only green spots were formed which were unable to grow into plants. It is known that in rice the regeneration ability is decreased or lost with the culture age (Jones, 1985). In subsequent transfers of cell clumps to regeneration media, a multistep procedure (Abe & Futsuhara, 1986; Zimny & Lorz, 1986) was adopted. The 3-4 weeks old cell clumps of newly established cell lines were transferred to MS with 0.05 mg/l NAA and 0.5mg/l BAP, N6 K2 and N6 K5 media (Table 2). The embryogenic calli were repeatedly trans ferred to same fresh media after every four weeks for a period of 3 months which resulted in regeneration 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 from cell suspensions of 'indica' rice.

Lee et al. (1989) successfully achieved reproducible and efficient regeneration from cell lines of immature embryos of IR-54, an 'indica' rice. In our study, immature embryos of all the tested genotypes were found to be highly responsive in solid media. However, only green spots were formed from the established cell suspension originated from calli of the immature embryos.

All the cell suspensions initiated from roots developed profused roots on regeneration media. In some jars, green roots which later turned to hairy white and finally to brown were formed. The multistep procedure (Abe & Futsuhara, 1986) was also employed but without success. These results emphasize the point that no general method exist for the establishment and subsequent regeneration of rice cell suspension. Therefore, the process of *in-vitro* morphogenesis still depends on genotype.

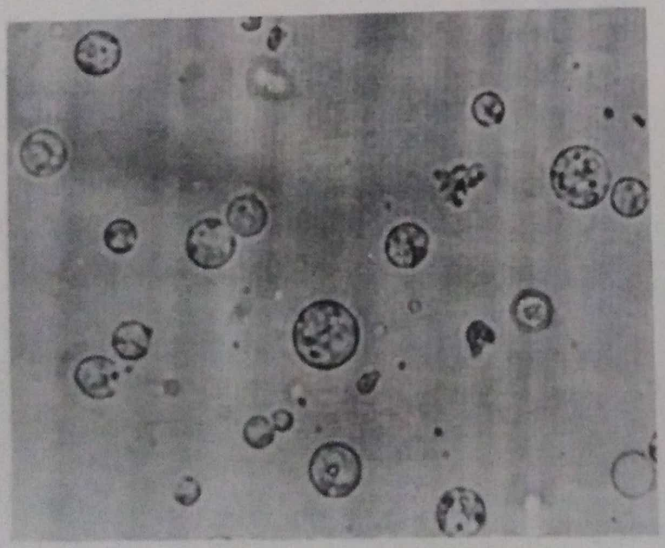

Figure 4. Protoplasts of DM-25-Inf before culturing on plates (X-200).

Protoplast Isolation

Several parameters were determined for the maximum yield of protoplasts from the newly established suspension line of DM-25-Inf. Maximum yield was observed from the cell material which was sub-cultured 3-4 days earlier from the stock culture in AA medium. Different pH's of the isolation medium (5.5 to 6.5) were tested and pH 6.0 was found to be optimum. Abdullah et al. (1986) had maintained pH of the isolation medium at 5.5 while Lee et al (1989) set it at 6.0. For culturing the protoplast, the droplet method of Thompson et al. (1986) and nurse culture technique (Kyozuka et al. 1987) was employed . Present experiments are directed to determine the suitable conditions for further division of protoplast to form microcalli and subsequently plant regeneration.

ACKNOWLEDGEMENTS

We wish to thank Mr. S. H. Zahid for his excellent technical assistance and

Mr. Javed Iqbal for his help in preparing the manuscript. Financial support partly provided by PARC-BOSTID project PAK-CS-PB-20.

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