PN-ABT-455 7.032
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International Rice Research Institute

Final Report for the Project

RICE SOMATIC EMBRYOGENESIS: ORIGIN, DEVELOPMENT AND SYNCHRONY, INHERITANCE AND GENETIC ANALYSIS

(AID Grant No.: 492-5542-G-IC-7064-00)

29 July 1987 - 31 October 1991

Title of Project:	Rice Somatic Embryogenesis: Origin, Development and Synchrony, Inheritance and Genetic Analysis
AID Grant No.:	492-5542-G-IC-7064-00
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Duration of Project:	July 29, 1987 - October 31, 1991

RICE SOMATIC EMBRYOGENESIS: ORIGIN, DEVELOPMENT AND SYNCHRONY, INHERITANCE AND GENETIC ANALYSIS
FINAL REPORT

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INTRODUCTION

The potential of somatic embryogenesis as a method for rapid in vitro multiplication of elite F_1 hybrid rices has been emphasized (Fujii et al, 1987). Possibly somatic embryogenesis can also be used as an experimental system for gaining more knowledge about embryo development and differentiation. Knowledge about embryo development and growth is fundamental, when developing methods for a high plant regeneration frequency and improved plant growth. Somatic embryogenesis in some extent may also be an advantageous for the protoplast culture, protoplast fusion and transformation studies, as has been the case with many other recalcitrant species (Vasil and Vasil, 1986). In recent years, there have been reports of successful in vitro somatic embryogenesis of many important graminaceous crops (Green, 1982). However, in rice, successful embryogenesis occurred rather sporadically and transiently (Abe and Futsuhara, 1985). In most experiments embryo was induced from callus on semisolid medium (Wang et al, 1990; Zapata and de Castro, 1988) and very recently from large clusters of cells in liquid medium (Ozawa and Komamine, 1989). Success in the induction of embryogenesis from single cell

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or small clusters of cells has not been possible. It is therefore important for physiological, biochemical and as well as for mass propagation, to establish embryogenic cell suspension culture from which embryos/plants could be regenerated at high frequency. However, the ability of somatic cell to an embryogenic cell cluster and the subsequent phases of in vitro embryogenesis largely depend on genotype, the explant source and culture conditions.

The objective of the investigation was to (a) screen a number of genotypes and identify a few which could be used as a model system in subsequent studies; (b) identification of factor which affect callus induction and embryogenic tissue initiation; (c) establishment of embryogenic cell suspension culture; (d) identification of factors which promote embryo development and plant regeneration; and (e) to extend the established system for possible in vitro mass propagation of elite F_1 hybrids.

EXPERIMENTAL STRATEGIES

Project 1: Development of Culture System for Certain Indica Rices

Experiment 1. Selection of genotype

The choice of appropriate genotype, explant source and culture medium critical for the success of somatic embryogenesis. Therefore, the experiment was carried out to identify suitable indica rice variety on the basis of embryogenic callus formation for establishing in vitro cultures. $MSCH_2$ medium (see Appendix 1) was chosen for

callus induction because preliminary experiments with different media composition indicated this medium is generally suitable for producing embryogenic callus. Using mature seeds, intervarietal variations in embryogenic callus formation were analyzed in ten indica varieties. Seventy to 100% of the plated mature seeds formed calli on the induction medium. Generally, two types of calli were produced, one type appeared creamy yellow and soft and contained distinct white globular somatic embryos on their surface (embryogenic calli) (Fig. 1). The other type of callus appeared light yellow to brown, hard and without any sign of organization (non-embryogenic calli). Based on this visual selection criteria, the frequency of somatic embryogenic capacity on solid medium varied with the varieties of rice (Table 1). The cultivars of Tetep and IR43 showed higher frequency of embryogenic capacity than other varieties. Therefore the IR43 was selected for further studies.

Experiment 2: Establishment of cell suspension cultures.

Cell suspension cultures provide a valuable tool for the improvement of crop plants. As the cell suspensions grow much faster than the callus cultures, therefore the chances of embryo production and plant regeneration are higher in cell suspension than in callus cultures. However, the compact and hard nature of embryogenic calli of most indica rices makes it very difficult to establish finely-

dispersed cell suspension cultures (Kyozuka et al, 1988). Therefore, the experiment was designed to establish cell suspension routinely. Primary callus (3 weeks old) used to initiate suspension cultures failed to grow in the liquid medium. In most cases, either the cultures formed vigorous roots and/or became brown. However, in some instances, only parts of the callus grew but it was extremely difficult to separate them from vigorously root forming cultures. In order to establish suitable embryogenic cell suspension, it was necessary to select the primary embryogenic callus and subculture in semisolid callus induction medium for at least six weeks (Table 2). These subcultured calli readily and repeatedly gave rise to stable cell suspensions. Initially the suspension cultures were composed mostly of different sizes of cell clusters. Through selective subculturing the small groups of cell, a homogeneous suspension of small group of cells were attained within two to three months. Individual cell of these clusters were densely cytoplasmic and isodiametric (Fig. 1b). Simultaneously, small globular embryos that developed at the surface of the aggregates were continuously released into the medium as free-floating structures (Fig. 1c).

Experiment 2A: Growth of the cell suspension.

Growth of the cells was monitored every other day from the day of subculture (day 0) to day 16. The growth of the cell suspension (FW and DW) as a function of time is shown

in Fig. 2. Between days 4 to 8, the doubling time for FW was approximately 2 days. Maximum FW was reached on day 14 and had increased 12-fold over the initial FW. Thereafter, the cells began to die as shown by the decrease in FW. A similar trend in DW was observed. The effect of various modifications of the nutrient medium on growth of the cells is shown in Table 3. Increasing the concentration of L-proline in the N_6P_2 medium (see Appendix 2) showed an increase growth. However, other treatments did not prove useful for the cultures.

Experiment 3: Organization and plant regeneration.

The concentration of 2,4-D had a significant influence on culture morphology. It has been reported that differentiation of somatic embryo required an auxin-free medium (Vasil and Vasil, 1985). Therefore, to induce further development of the somatic embryos, suspension cultures were transferred to N_6P_2 medium that contained 0 to 0.2 mg/l 2,4-D. Although further organization was observed (Fig. 1d-f), complete embryo development was not detected. The cells grew and produced a large number of clumps with a few green shoots when transferred to the light.

For further examination of the factors controlling regeneration, attempts were made to induce the development of embryos or shoots directly from cells in suspension cultures. N_6 medium supplemented with 30 g L^{-1} sucrose, and 0.5 mg L^{-1} each of BAP and NAA supported shoot regeneration

(8.67 ± 2.08 shoots/50 mg FW cells) but not MS medium. The varied response of cells in MS and N_6 liquid media suggests that the amount and form of nitrogen play an important role in obtaining differentiation from rice cells. Shoot formation was improved when the cells were transferred to 1/2 strength N_6 medium (Table 4) cells that were transferred to 1/4 strength N_6 medium showed retarded growth, and the shoots that were produced were very weak. Different concentrations of the hormonal supplements of the half-strength N_6 regeneration medium were tested (Table 5). The addition of 0.5 mg L^{-1} each of BAP and NAA was optimal for regeneration. Although addition of cytokinin was essential for efficient differentiation, high concentrations of cytokinin did not improve differentiation. Although many green shoots (Fig. 1g) were obtained after prolonged (15 months) culture, few developed into plants (Fig. 1h, I).

The procedure for establishing embryogenic cell suspension were likewise successfully extended to other indica cultivars namely IR72, Tetep, Dular, etc.

Project 2: Development of Culture System for Elite Indica x Indica F_1 Hybrids

Expt. 1: <u>Embryogenic callus induction and establishment of cell suspension</u>.

Embryogenic callus was produced on $MSCH_2$ media using dehulled mature seeds of four F1 hybrids. All the hybrids produced embryogenic callus (Table 6). Primary callus from single seed was selected and subcultured on semisolid callus

induction medium for certain period. Actively growing secondary callus was used to initiate embryogenic cell suspensions.

Expt. 1A: Effect of carbohydrate source on the establishment of embryogenic cell suspension.

The most commonly used carbohydrate for plant tissue or cell culture is sucrose and most plant culture media have it as the sole carbohydrate source. It is assumed that sucrose is the best carbohydrate in cell culture media because it is the main transport form of carbohydrate in most species. However, from previous experience with indica rices, it was observed that although sucrose could be used to develop embryogenic cell suspension but sucrose as a carbohydrate to some extent limit the proembryogenic clumps to differentiate into plants. therefore, an alternative carbohydrate source such as maltose was compared to develop embryogenic cell suspensions. Secondary embryogenic callus that transferred to R2 (see Appendix 3) liquid medium containing maltose supported to establish embryogenic cell suspension of all the four F1 hybrids (Table 5). The callus easily adapted and grew vigorously in maltose containing liquid medium. The cell clusters easily dissociated in the liquid medium and suspension became established within two months of culture. When maintained on a weekly subculture schedules, the cultures showed a four to five-fold increase in fresh weight. On the contrary, cell clusters in the sucrose containing liquid R2 medium often got browning or formed

roots. However, some of the cell clusters continued to grow without forming roots. The growth rate of these cell clusters was rather poor, and repeated subculture did not improve growth and dissociation. Thus the subsequent studies was carried out with only the cell suspensions growing in maltose containing medium.

Expt. 1B: Plant regeneration from cell suspension of F1 hybrids: effect of cytokinin.

Plant regeneration experiment was carried out using IR58025A/IR54742-22-19-3R (IR65488H) as test material.

Initially, equal amount of cells were distributed to surface of the semisolid MS, N6 or R2 regeneration media and found that R2 media is superior than that of MS and N6 media in producing green plants (data not shown). Thus R2 to optimize the regeneration condition, R2 media was supplemented either with kinetin (K) or benzylaminopurine (BAP). The results obtained showed that kinetin is far more superior than that of BAP (Table 7). Using this system more than thousands of plants were regenerated and will be evaluated in the field to see phenotypic variation. A schematic diagram (Fig. 3) for plant regeneration from cell suspension of F1 hybrids are given.

FINDINGS AND LIMITATIONS

- Embryogenic callus could be produced using mature embryo as explant from most of the indica cultivars as well as from indica x indica F1 hybrids.
- N_6 and R_2 liquid media supplemented either with sucrose or maltose are suitable for establishment of embryogenic cell suspension, however R_2 medium couple with maltose is more effective.
- Large number of plant regeneration readily be achieved by using R_2 medium that contain maltose and kinetin.
- Mode of plant regeneration seems to be via secondary embryogenesis and/or from the clump of cells, thus limiting the concept of artificial seed production.

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Table 1. Frequency of embryogenic callus (E-callus) from mature seeds of various indica rice[1].

Variety	Percent E-callus
Tetep	70
IR72	34
IR43	55
IR42	51
IR64	44
IR56*	23
IR74	27
IR24	45
IR58*	36
IR28	42

[1]Basal medium is MS containing 2 mg/l 2,4-D, 1.0 g/l CH, 30 g/l sucrose and 8 g/l agar.

Table 2. Effect of recurrent selection of embryogenic callus and subculture on the establishment of cell suspension.[1]

	Primary Callus (3-wk-old)	Sub-culture 1	Sub-culture 2	Sub-culture 3	Sub-culture 4	Sub-culture 5
Response	(-)	(-)	(-)	(+)	(+)	(+)

[1]Subculture was performed every two weeks interval.

Table 3. Effect of various modifications of the nutrient medium on the changes of growth of cell in suspension culture. Six samples from three separate flasks were used to measure growth (P=proline, CH=casein hydrolysate).

Nutrient Medium	FW (mg/ml)	DW (mg/ml)
N_6P_2 (control)	50.13	6.5
Without proline	40.14	5.37
With 50 mM proline	55.3	7.81
With 1.0 g/l CH	35.79	5.64
With 4.0 mg/l 2,4-D	46.81	6.39
With 1.0 mg/l 2,4-D	49.5	6.26
Half strength of the control	40.95	6.14
Double strength of the control	36.34	5.54
Without ammonium salt	25.36	5.54
With 3x ammonium salt	30.80	4.48
Without potassium nitrate	25.62	3.45
With 3x potassium nitrate	21.31	3.22

Table 4. Effect of salt concentration in liquid medium on shoot regeneration from cell suspensions of indica rice.

Regeneration medium[1]	No. of green shoots/ 50 mg FM of cells[2]
N_6	10.0 ± 1.73
1/2 N_6	18.33 ± 3.5
1/4 N_6	14.67 ± 1.52

[1]Supplemented with NAA (0.5 mg L^{-1}), BAP (0.5 mg L^{-1}) and sucrose (30 g L^{-1}).

[2]Mean of three replications.

Table 5. Effect of NAA and BAP on shoot regeneration from cell suspension of indica rice.[1]

BAP (mg L^{-1})	NAA (mg L^{-1})	No. of green shoots/ 50mg FM of cells[2]
1.0	0.5	17.33 ± 3.05
1.0	0.1	18.0 ± 5.29
0.5	0.5	19.0 ± 2.94
0.5	0.1	16.67 ± 5.03
0.1	0.1	8.67 ± 1.53
0	0	3.33 ± 1.53

[1]Basal medium is half-strength N_6 containing 30 g L^{-1} sucrose.

[2]Mean of 3 replications.

Table 6. Culture response of four indica x indica F_1 hybrids.

Hybrids	Embryogenic callus initiation[1]	Suspension culture[2]	Plant regeneration[3]
IR58025A/ IR29723-143-3-2-1R (IR64615H)	+	+	NT
IR58025A/ IR9761-19-1R (IR64611H)	+	+	NT
IR62829A/ IR29723-143-3-2-1R (IR64616H)	+	+	NT
IR58025A/ IR54742-22-19-3R (IR65488H)	+	+	+

NT = not tested

[1]Basal medium is MS containing 2 mg/l 2,4-D, 1.0 g/l CH, 30 g/l sucrose and 8 g/l agar ($MSCH_2$).

[2]Basal medium R_2 containing 1.0 mg/l 2,4-D, 5 mM L-proline and 30 g/l maltose.

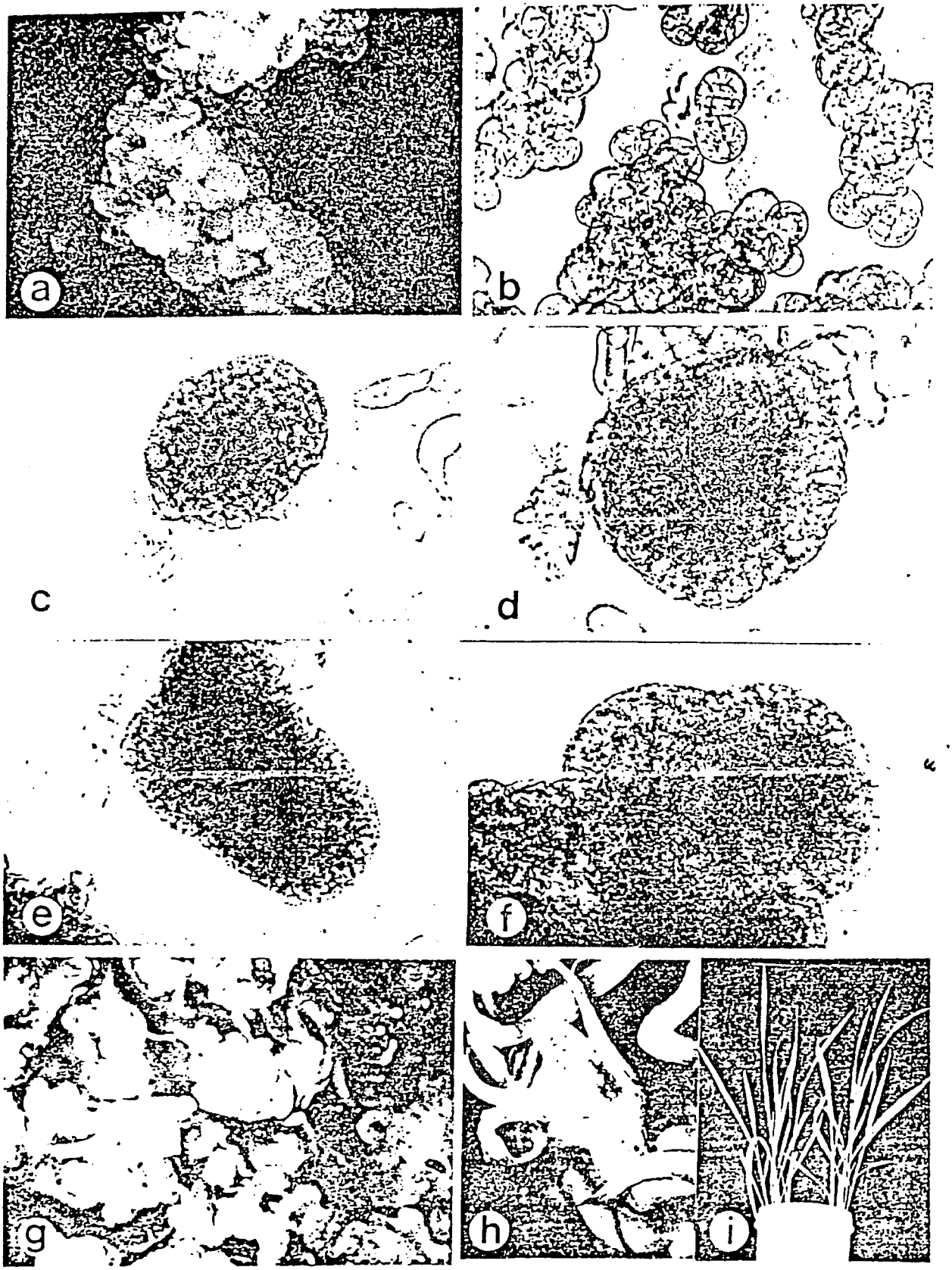
[3]Basal medium R_2 containing 5 mg/l K, 1 mg/l NAA, 30 g/l maltose and 8 g/l agarose.

Table 7. Effect of cytokinins on plant regeneration from cell suspension cultures of F_1 hybrids.

Regeneration medium[a]	No. of plants/ gm of cells[b]
R_2K	192
R_2B	146

[a]Regeneration medium supplemented with 1.0 mg/l NAA, 5 mg/l Kinetin (K) or benzylaminopurine (B), 30 g/l maltose and 8 g/l agarose.

[b]Average of two experiments.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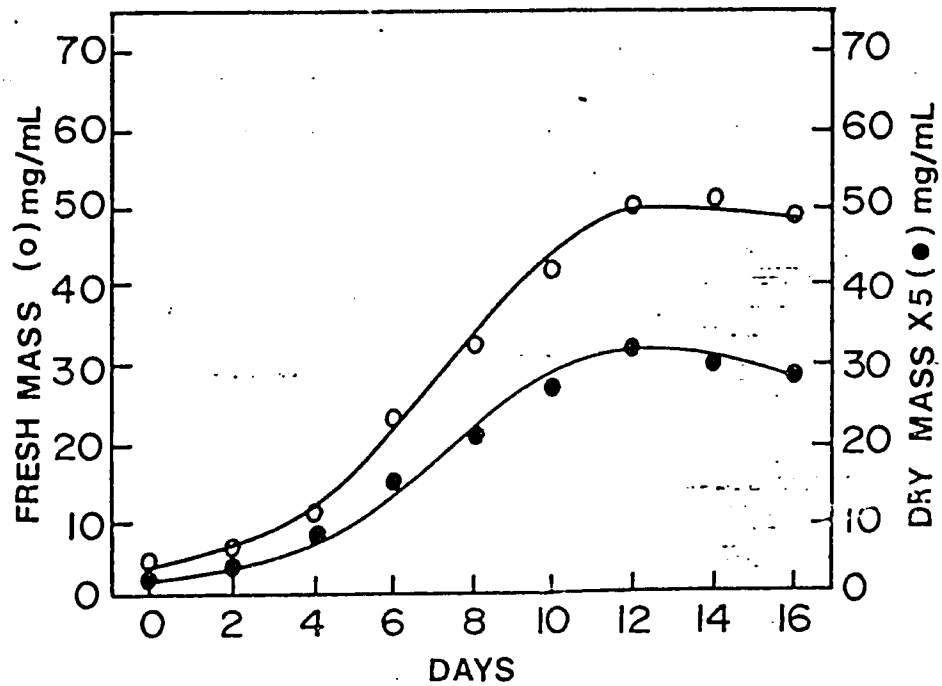
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16

FIGURE LEGENDS

Fig. 1a-i. Plant regeneration from cell suspension cultures of indica rice.

a. Embryogenic callus used to initiate embryogenic cell suspensions.

b. Cells from the embryogenic cell suspension culture.

c. Early globular stage somatic embryo.

d. Late globular stage somatic embryo with a single epidermal layer at the outer surface.

e,f. Advanced organization of somatic embryos.

g,h. Green shoots and plantlets regenerated in liquid medium.

i. Regenerated plants in the greenhouse.

Fig. 2. Growth curve of cell suspension cultures of indica rice grown in N_6P_2 liquid medium.

Fig. 3. Schematic diagram for plant regeneration of indica x indica F_1 hybrids from embryogenic cell suspension.

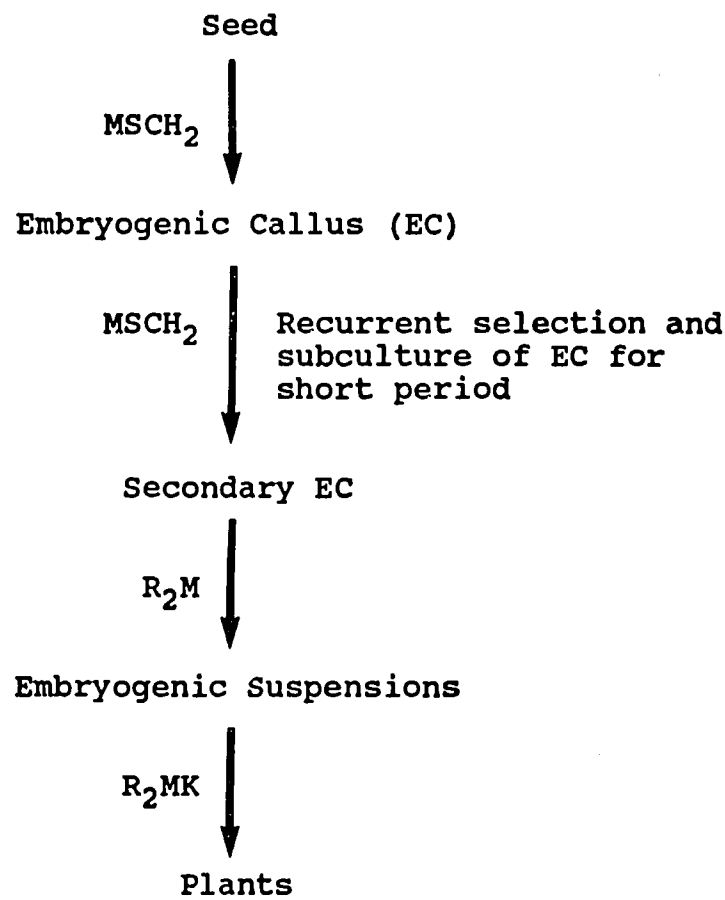

Fig. 3. Protocol for indica x indica F_1 hybrids embryogenic cell suspensions.

Appendix 1. Composition of $MSCH_2$ medium.

	Amount (mg/l)
Macronutrients	
KNO_3	1900
NH_4NO_3	1650
$MgSO_4.7H_2O$	370
$CoCl_2.2H_2O$	440
KH_2PO_4	170
Micronutrients	
KI	0.83
H_3BO_3	6.2
$CoCl_2.2H_2O$	0.025
$Na_2MoO_4.4H_2O$	0.25
$CuSO_4.5H_2O$	0.25
$ZnSO_4.7H_2O$	8.6
$MnSO_4.4H_2O$	22.3
$FeSO_4.7H_2O$	27.85
Na_2EDTA	37.25
Vitamins	
Myo-Inositol	100
Nicotinic acid	0.5
Thiamine HCl	0.1
Pyrodoxine HCl	0.5
Sucrose	30,000.0
Casein hydrolysate	1000
2,4-D	2.0
Agar	8,000

Appendix 2. Composition of N_6P_2 medium.

	Amount (mg/l)
Macronutrients	
KNO_3	2830
$(NH_4)_2SO_4$	463
K_2HNO_4	400
$MgSO_4.7H_2O$	185
$CaCl_2.2H_2O$	166
Micronutrients	
KI	0.8
H_3BO_3	1.6
$MnSO_4.4H_2O$	4.4
$ZnSO_4.7H_2O$	1.5
$FeSO_4$	27.8
Na_2EDTA	37.3
Vitamins	
Myo-Inositol	100
Nicotinic acid	0.5
Thiamine HCl	1.0
Pyridoxine HCl	0.5
Sucrose	3000.0
Proline	50 mM
Glycine	2.0
2,4-D	2.0

Appendix 3. Composition of R_2 medium.

	Amount (mg/l)
Macronutrients	
KNO_3	4044
$(NH_4)_2SO_4$	330
NaH_2PO_4	276.02
$MgSO_4.7H_2O$	246.5
$CaCl_2.2H_2O$	110.99
Micronutrients	
$CoCl_2.6H_2O$	0.025
$ZnSO_4.7H_2O$	2.0
$MnSO_4.H_2O$	0.25
$CuSO_4.3H_2O$	0.125
H_3BO_3	3.0
$NaMoO_4.2H_2O$	0.25
$FeSO_4$	12.5
Na_2EDTA	16.5
Thiamine	1.0
Proline	5 mM
Sucrose/Maltose	3000.0
2,4-D	1.0

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International Rice Research Newsletter Vol. 16 No. 2 (April 1991) : 9-10

Breeding methods – tissue culture

Embryogenic cell suspension and plant regeneration from protoplasts of indica rice

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Cell suspension established from anther-derived callus of indica rice cultivar IR43 was used for protoplast isolation and culture.

Callus was induced following the standard anther culture technique developed at IRRI: Anthers inoculated individually in plastic petri dishes (60 x 15 mm) containing 6 ml of SK-1 medium, and cultures incubated in the dark at 25 ± 1 °C for 1 mo.

Embryogenic calli (approximately 2 g) were selected and placed in 125-ml Erlenmeyer flasks containing 30 ml of liquid AA medium to initiate suspension cultures. The cultures were placed on a gyrotary shaker (120 rpm) in the dark at 25 ± 1 °C. Liquid medium was replaced every 5-6 d. After 3-4 mo, the actively dividing embryogenic cell suspension established was maintained by weekly subculturing at 1:4 dilution (suspension inoculum:fresh medium).

To isolate protoplasts, 3- to 4-d-old cells were incubated for 4 h in a solution containing 1.0% Cellulase Onozuka RS, 0.2% Pectolyase Y-23, 5 mM MES, CPW salts, and 13% mannitol at pH 5.6. The protoplasts were sieved through 45- to 25-µm nylon mesh, collected by centrifuging at 100 x *g* for 5 min, and washed 3 times in CPW salts containing 13% mannitol.

Protoplasts were cultured in 60- x 15-mm plastic petri dishes containing 3 ml MS1 medium at 1-2 x 10^5 pp/ml, and maintained in the dark at 25 ± 1 °C. After 3-4 wk, the agarose layer was cut into four blocks and transferred to a fresh medium without glucose but supplemented with 3.0% sucrose.

For plant regeneration, 100 calli about 4-5 mm in diameter were transferred to test tubes containing 20 ml of MSYo medium and kept under 12 h dark/light photoperiod at 25 ± 1 °C.

Media compositions (SK-1, AA, MS1, MSYo) and growth substances used for different steps of the cultures are given in the table.

The type of callus used (see figure, 1) was highly suitable for developing a suspension culture composed of small groups of cytoplasmic and starch-containing embryogenic cells (2). Protoplasts isolated from this suspension displayed dense cytoplasm (3).

The first division was observed after 4 d of culture (4); multicellular structures were formed after 8-10 d of culture (5). Within 3 wk of culture, most of the structure underwent further growth leading to the direct formation of recognizable globular embryoids (6). Colony formation was counted at this stage. About 0.5-0.75% plated protoplasts formed colonies. Upon transfer to

Media composition.

Constituent	Amount (mg/liter)			
	SK-1	AA	MS1	MSYo
KNO_3	3,150	-	1,900	1,900
NH_4NO_3	-	-	1,650	1,650
KH_2PO_4	540	170	170	170
$CaCl_2 \cdot 2H_2O$	150	440	440	440
$MGSO_4 \cdot 7H_2O$	185	370	370	370
$FeSO_4 \cdot 7H_2O$	27.85	27.85	27.85	27.85
Na_2EDTA	37.25	37.25	37.25	37.25
KCl	-	2,940	0	0
KI	1.0	0.83	0.83	0.83
$CoCl_2 \cdot 6H_2O$	-	0.025	0.025	0.025
$Na_2MoO_4 \cdot 2H_2O$	0.25	0.25	0.25	0.25
$CuSO_4 \cdot 7H_2O$	0.025	0.025	0.025	0.025
$ZnSO4 \cdot 7H_2O$	10	8.6	8.6	8.6
$MnSO4 \cdot 4H_2O$	22.3	22.3	22.3	22.3
H3BO3	6	6.2	6.2	6.2
Inositol	100	100	100	100
Nicotinic acid	2.5	0.5	0.5	0.5
Pyridoxine-HCl	2.5	0.5	0.5	0.5
Thiamine-HCl	2.5	0.1	0.1	0.1
Glycine	2.0	75.0	2.0	2.0
L-glutamine	-	877	-	-
L-aspartic acid	-	266	-	-
L-arginine	-	228	-	-
Sucrose	40,000	30,000	-	30,000
Glucose	-	-	90,000	-
Casein hydrolysate	500	-	-	-
2,4-D	0.75	2.0	1.0	-
NAA	2.5	-	-	1.0
Kinetin	0.75	0.2	-	1.0
GA_3	-	0.1	-	-
Agar	8,000	-	-	8,000
Agarose	-	-	5,000	-
pH	5.8	5.8	5.8	5.8

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Running title:
Plant Regeneration from Indica Rice

J. Plant Physiol. Vol. . pp. - (1991)

7.032

Plant Regeneration from Long-Term Cell Suspension Cultures of Indica Rice (*Oryza sativa* L. cv. IR43)

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Received May 7, 1991 · Accepted July 24, 1991

Summary

Embryogenic callus initiated from mature seeds of the Indica rice cultivar IR43 was used to establish embryogenic cell suspension cultures. Suspension cultures were coomposed of cells that were densely cytoplasmic and potentially embryogenic. Globular somatic embryos were continuously produced in N6 liquid culture medium containing 2.0 mg L^{-1} 2,4-D. These embryos underwent further organized growth upon transfer to N6 medium with less or without 2,4-D. A large number of green shoots were obtained upon transfer to a half-strength liquid N6 medium containing 0.5 mg L^{-1} NAA, 0.5 mg L^{-1} BAP and 30 g L^{-1} sucrose. The cultures showed sustained regeneration capacity for 15 months.

Key words: Oryza sativa L., Indica, embryogenic cell suspension, plant regeneration.

Abbreviations: BAP = 6-benzylaminopurine; CH = Casein hydrolysate; 2,4-D = 2,4-dichlorophenoxyacetic acid; IAA = indole-3-acetic acid; NAA = 1-naphthaleneacetic acid.

Introduction

A culture system for embryogenic cell suspension and subsequent plant regeneration of rice could be used in the genetic modification and mass propagation of this important crop.

Establishment of cell suspension cultures of rice and subsequent plant regeneration from relatively large clusters of cells via organogenesis (Zimny and Lorz, 1986) and via embryogenesis (Ozawa and Komamine, 1989) have been reported. However, the modern indica rice cultivars grown widely in tropical regions, have been classified as non-responding (Kyozuka et al., 1988). Although morphogenic cell suspensions of an indica cultivar (IR54) have been established and used as the source of protoplasts (Lee et al., 1989), the induction of somatic embryogenesis from improved Indicas grown in cell suspension culture has not been reported.

The establishment of a long-term (15-month-old) embryogenic cell suspension of an improved Indica genotype, IR43, in which embryos develop and plants are regenerated in liquid medium is reported in this paper.

Materials and Methods

Callus Initiation

Dehulled mature seeds of IR43 (Indica type) were surface-sterilized in 70% ethanol for 1 min and in 0.1% $HgCl_2$ for 10 min followed by three washes in sterile distilled water. The seeds were then cultured onto 20 mL semisolid medium containing MS salts and vitamins (Murashige and Skoog, 1962) supplemented with 2.0 mg L^{-1}, 2,4-D, 1.0 g L^{-1} CH, 3.0% sucrose and 0.8% Difco Bacto agar (MSCH2), pH 5.8. Ten seeds were placed in each Falcon petri dish (100 × 15 mm). The medium was sterilized at 120 °C for 15 to 20 min. Cultures were maintained at 25 ± 1 °C in the dark. After four weeks, the calli were subcultured in the same medium for another three weeks before initiation of suspension culture.

Initiation and Maintenance of Suspension Culture

Approximately 2.0 g fresh mass (FM) of callus was placed into each 125-mL Erlenmeyer flasks containing 40 mL of liquid B5 medium (Gamborg et al., 1968) without $(NH_4)_2SO_4$ but supplemented with 300 mg L^{-1} L-glutamine, 1.0 mg L^{-1} 2,4-D and 3.0% sucrose. The medium was adjusted to pH 5.8 and filter sterilized. The cultures were incubated in the dark at 25 ± 1 °C on a rotary shaker (120 rpm), and the medium was replaced every 7 d. After 5 to 6 weeks, the creamy yellow, proliferating calli (referred to as adaptive calli) were transferred and grown for four weeks in liquid medium containing MS salts and vitamins, supplemented with 1.0 mg L^{-1} 2,4-D and 3.0% sucrose (MS1), then transferred to N_6 medium (Chu et al., 1975) supplemented with 2.0 mg L^{-1} 2,4-D, 50 mM L-proline, and 3.0% sucrose (N_6P_2), pH 5.8. Established cell suspensions in N6P2 medium were maintained by weekly subculture at a dilution ratio of 1:4 inoculum/fresh medium.

Growth Measurements

Well-mixed samples (100 mg FM) were transferred to 125 mL Erlenmeyer flasks containing 25 mL of liquid N6P2 medium. Growth was monitored every other day from the day of subculture (day 0) to day 16. To determine fresh mass, cells from 5 mL samples were collected on a filter paper, drained under vacuum, transferred onto preweighed aluminum foil, and immediately weighed again. Dry mass (DM) was measured after drying the cells at 60 to 65 °C for 18 h.

Organization and Plant Regeneration

To encourage further development of somatic embryos or to regenerate plants from small embryos that were continuously produced in the suspension cultures, the following procedure was followed. Cells and embryos were harvested from embryogenic cell suspensions and pipetted into 16 mL centrifuge tubes. The tubes were spun at 100 × g for 5 min, and the supernatant was discarded. The cells were then washed twice with the same medium in which the cells were subsequently cultured. The washed cells (50 mg FM) were resuspended in a tube (30 × 200 mm) containing 20 mL of liquid regeneration medium (see Tables 1 and 2). The tubes were then rotated horizontally at 10 to 15 rpm at 25 ± 1 °C under diffuse light. After 14 d, the inoculum from the tubes was distributed to plastic Petri dishes and the number of green shoots were counted.

Regenerated shoots were transferred into test tube containing 20 mL of ½ strength MS or N_6 solidified medium with or without 1 mg L^{-1} IAA, and 0.8% (w/v) Difco Bacto agar for development of the root system. After 3 to 4 weeks, rooted plants were transferred to soil in pots and were grown in a greenhouse until maturity.

Results

Initiation of Callus

Calli were induced from 70 to 80% of the mature embryo explants on MSCH2 medium within two weeks of culture. Two types of calli were produced. One type appeared creamy yellow and soft and contained distinct white globular somatic embryos on their surface (embryogenic calli).

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Sporadically, some of the organized structures developed into a cup-shaped scutellum with a tubular coleoptile. The other type of callus appeared light yellow to brown, hard and without any sign of organization (non-embryogenic calli). Upon transfer to a fresh medium, the latter type failed to grow. The embryogenic callus or those bearing small globular embryos continued to grow in the same medium without further development (Fig. 1a). Actively growing callus of this type was used for the initiation of cell suspensions.

(2) Figs. 1a–i

Initiation and Maintenance of Cell Suspensions

Several attempts to initiate cell suspensions in liquid MSCH2 and several other media were not successful (data not shown). Either the culture became rooty or necrotic and eventually died. However, some cell clusters continued to grow without root formation in modified liquid B5 medium; individual cells of these clusters were round and densely cytoplasmic. The growth rate of these cell clusters was rather poor, and repeated subculture did not improve growth. Transfer of these adapted cell clusters to liquid MS1 medium resulted in vigorous growth with large aggregates of cells. Occasionally, small groups of round, densely cytoplasmic cells with a thin cell wall appeared. Growth and dissociation of the cell aggregates was further improved when the cell aggregates were transferred to liquid N_6P_2 medium. After a few weeks of weekly subculture, fast-growing cell lines composed mostly of highly cytoplasmic and spherical cells were obtained (Fig. 1b). Simultaneously, small globular embryos that developed at the surface of the aggregates were continuously released into the medium as free-floating structures (Fig. 1c). Further development of the embryos in the maintenance medium was not observed.

(3) Fig. 2

The growth of the cell suspension (FM and DM) as a function of time is shown in Fig. 2. Between days 4 to 8, the doubling time for FM was approximately 2 d. Maximum FM was reached on day 14 and had increased 12-fold over the initial FM. Thereafter, the cells began to die as shown by the decrease in FM. A similar trend in DM was observed.

The growth of the cells was kept relatively stable over a 15-month period, following weekly subculture (see Materials and Methods). Additions of CH (0.5 to 2.0 g L^{-1}) in the N_6P_2 medium to improve the morphology of the cultures did not prove useful, although an additional amount of L-proline increased growth (data not shown).

Organization and Plant Regeneration

To induce further development of the somatic embryos, suspension cultures were transferred to N_6P_2 medium that contained 0 to 0.2 mg L^{-1} 2,4-D. Although further organization was observed (Figs. 1d–f), complete embryo development was not detected. The cells grew and produced a large number of clumps with a few green shoots when transferred to the light.

For further examination of the factors controlling regeneration, attempts were made to induce the development of embryos or shoots directly from cells in suspension cultures. N_6 medium supplemented with 30 g L^{-1} sucrose, and 0.5 mg L^{-1} each of BAP and NAA supported shoot regeneration (8.67 ± 2.08 shoots/50 mg FM cells) but not MS medium. Shoot formation was improved when the cells were transferred to ½-stength N_6 medium (Table 1). Cells that were transferred to ¼ strength N_6 medium showed retarded

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④ Table 1

⑤ Table 2

Different concentrations of the hormonal supplements of the half-strength N_6 regeneration medium were tested (Table 2). The addition of 0.5 mg L^{-1} each of BAP and NAA was optimal for regeneration. Increased BAP concentrations did not improve shoot differentiation. Although many green shoots (Fig. 1 g) were cotained after prolonged (15 months) culture, few developed into plants (Figs. 1 h, i).

Discussion

Callus type and the selection of cell clusters that were able to adapt to the specific liquid medium were essential in the establishment of an embryogenic cell suspension culture of IR43. The importance of these selection procedures has also been demonstrated by other workers (Abdullah et al., 1986; Luhrs and Lorz, 1988; Shillito et al., 1990. Redway et al., 1990). Numerous embryogenic calli were ated in liquid media. Some of these calli adapted to the ied liquid B5 medium; however, this medium did not s fast growth. Upon transfer of the adaptive cell cluster: uid N_6P_2 medium, the cells grew and continuously ed globular embryos for a prolonged period without hoot formation capacity.

The concentration of 2,4-D had a signi nfluence on culture morphology. As described earlie evelopment of globular somatic embryos took place presence of 2,4-D. It has been reported that further ntiation of somatic embryo required a hormone-free n (Vasil and Vasil, 1985). In our studies, a higher frequ differentiation occurred when the cells were transfe a medium that contained both cytokinin and auxi ilar results were also observed with wheat callus (Carman, 1988).

The varied response of cells in MS anc quid media suggests that the amount and form of n play an important role in obtaining differentiatio rice cells. Cells differentiated in liquid N_6 medium t in liquid MS medium. Similar effects of inducing n genesis via somatic embryogenesis in protoplast-deri lus of rice were reported (Abdullah et al., 1986).

In an attempt to maximize regeneration, it was found that the concentration of the salts in N_6 medium has a marked influence on differentiation. The regeneration frequency was almost twice that in ½ N_6 salts than in full strength N_6 (Table 1). This suggests that full strength N_6 medium contains excessively high components that inhibit embryo differentiation. One such component may be the total inorganic nitrogen level in N_6 medium (35 mM). For the same reason, the complete inhibition of differentiation in MS media may also be caused by supraoptimal levels (60 mM) of inorganic nitrogen salts.

Various combinations and concentrations of hormones that have been used to influence the regeneration capability showed little effect. Although addition of cytokinin was essential for efficient differentiation, high concentrations of cytokinin did not improve differentiation. The highest frequency of shoot differentiation was obtained in ½ strength of N6 medium supplemented with NAA (0.5 mg L^{-1}), BAP (0.5 mg L^{-1}), and sucrose (3.0 %). A similar observation on the relatively low requirement of cytokinin for regeneration was also made by Ozawa and Komamine (1989).

To our knowledge, this is the first report on the regeneration of plants in liquid medium from long-term cultures of modern Indica rice.

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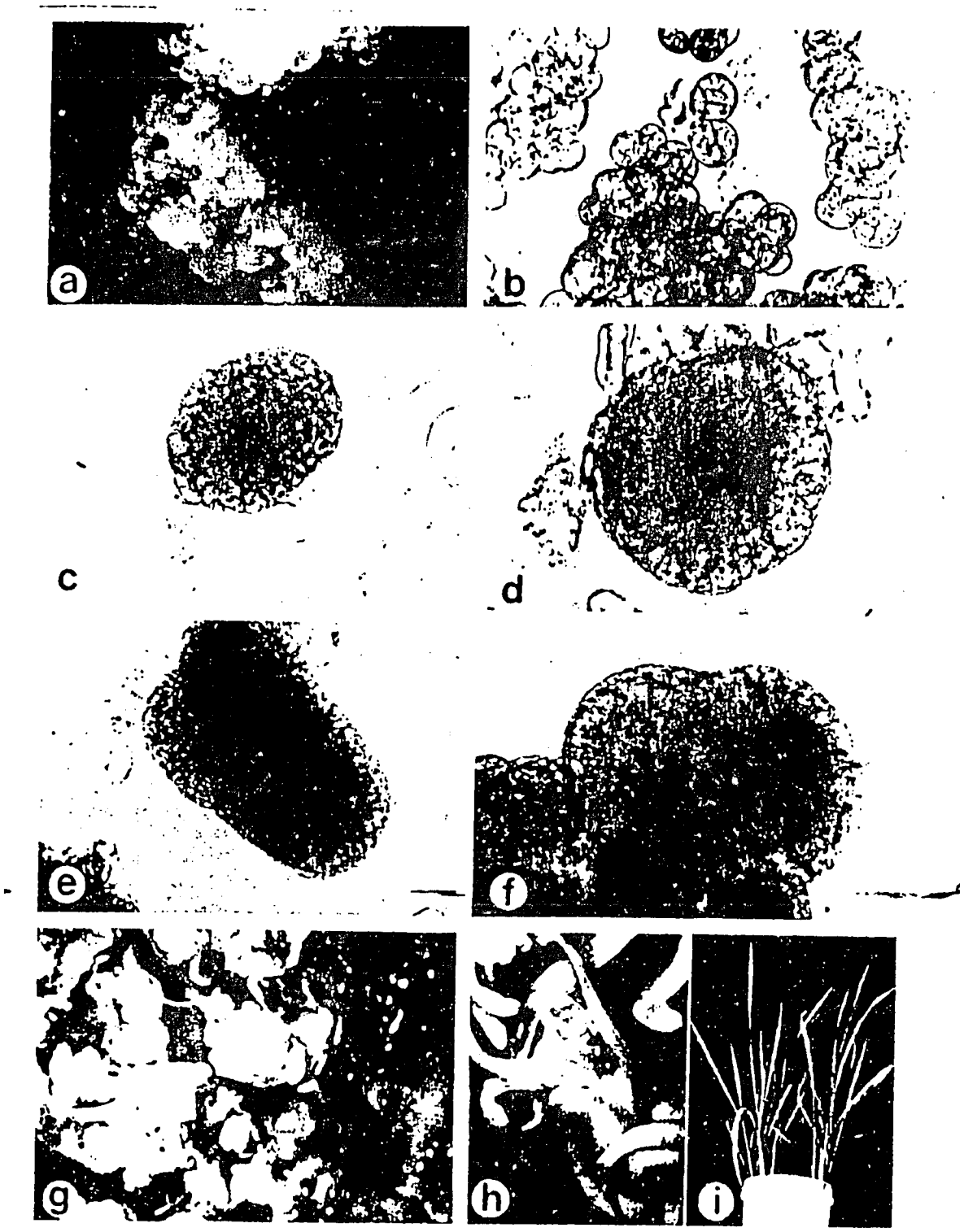

Figs. 1 a–i: Plant regeneration from cell suspension cultures of Indica rice. a. Embryogenic callus used to initiate embryogenic cell suspensions. b. Cells from the embryogenic cell suspension culture. c. Early globular stage somatic embryo. d. Late globular stage somatic embryo with a single epidermal layer at the outer surface. e, f. Advanced organization of somatic embryos. g, h. Green shoots and plantlets regenerated in liquid medium. i. Regenerated plants in the greenhouse. (2)