RESULTS

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1. Initiation of callus

Initiation of callus was observed from mature seed explants inoculated on L.S. medium supplemented with different concentrations of 2,4-D from 5th day onwards. It has been observed that the callus was derived from the scutellum (Plate 1a). Among different concentrations of 2,4-D tested, 2.5 mg/l of 2,4-D brought about the maximum percentage of callus induction (72.3%, Table 3)

Table 3 : Effect of different concentrations of 2,4-D on percentage of callus induction in rice. (Data recorded after 30 days)

LS ± Concentration of 2,4-D (mg/l)	% of explant with calli induction
0.0	*0.0±0.00
0.02	0.0±0.00
0.05	2.4 ±0.42
0.1	7.81 ±0.2
0.5	12.25 ±0.81
1.0	22.60 ±0.1
2.0	41.3 ±0.41
2.5	72.3 ±0.8
3.0	38.7 ±0.21
3.5	35.1 ±0.4

* Mean ± S.E. values of three independant experiments with four replicates.

Plate 1 a) Mature rice embryo showing callus induction from scutellum (arrow) on L S + 2 5 mg/l of 2,4-D

b) One month old callus of rice in callus induction medium

Bar = 1 cm

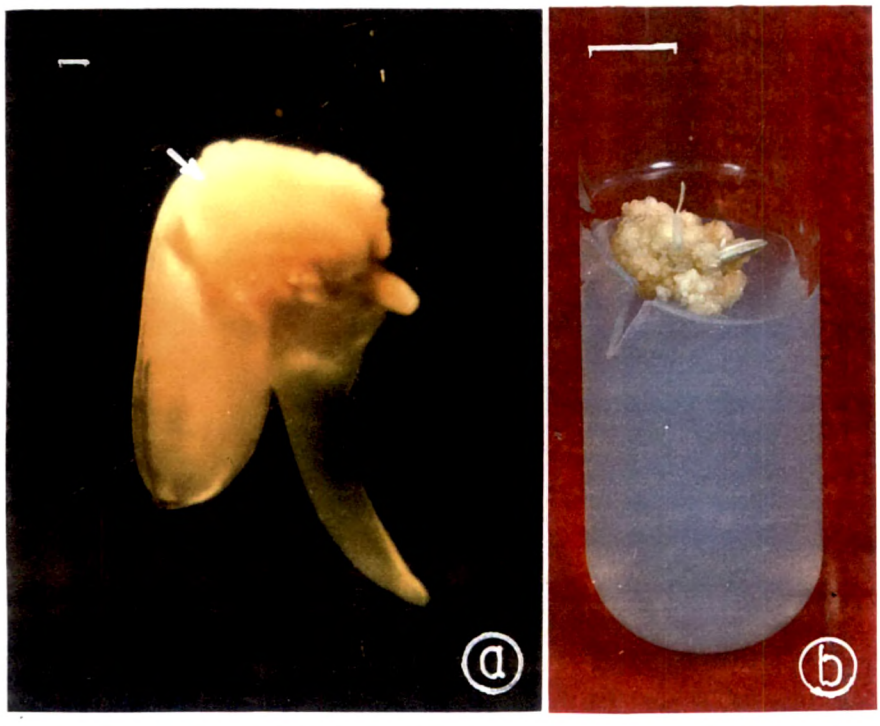


Plate 1

Plate 2 (a-h) Four week old callus of rice under different concentrations of NaCl (0-300 mM)

Bar = 1 cm

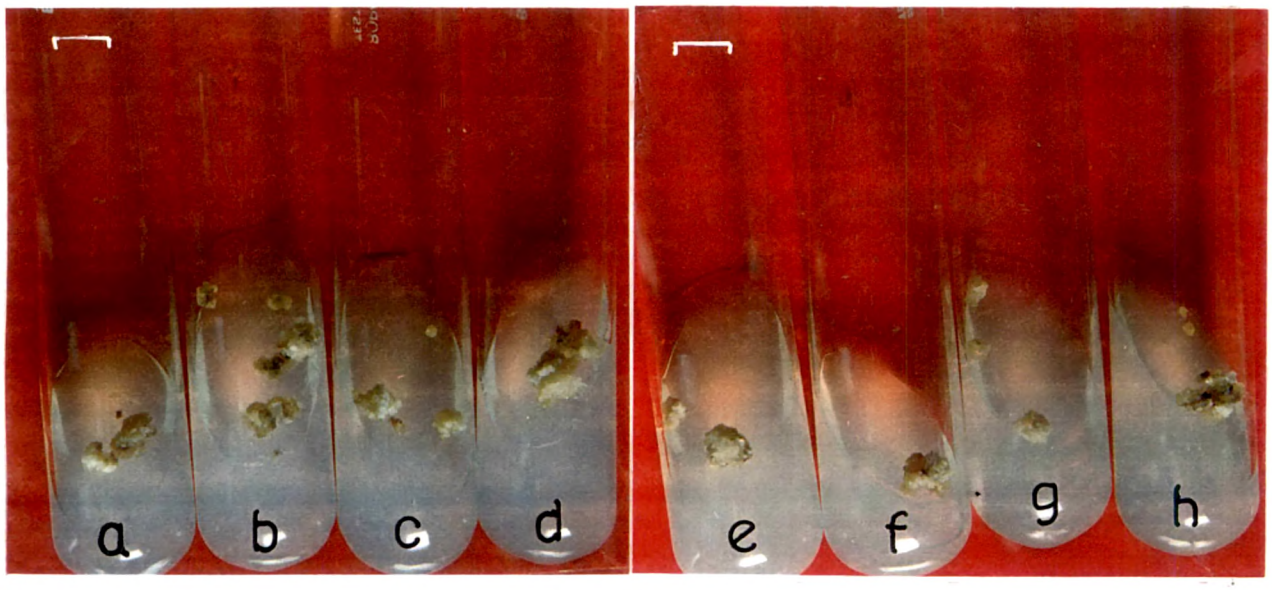


Plate 2

and any further increase in the level of 2,4-D failed to increase the percentage of callus induction. The callus thus derived was a small yellowish mass by the end of thirty days of incubation (Plate 1b). This callus was seperated and used for further subcultures.

2. Growth Analysis

The induced callus was then subcultured regularly at an interval of 15 days. The growth of the callus showed a typical pattern registering maximum growth at the end of fourth week and reached a stationary phase thereafter. Incorporation of varying concentration of NaCl (0-300 mM) did not alter the pattern of growth, but it led to a reduction in growth and the growth inhibition was found to be concentration dependent (Table 4, Plate 2 a-h).

Table 4 : Effect of different concentrations of NaCl on dr. wt(mg) of rice callus.

Con. of NaCl (mM) used	Incubation periods in weeks			
	0	2	4	6
0	*20 ±0.2^{a}	43 ±0.3^{a}	82 ±0.7^{a}	80 ±0.21^{a}
50	19 ±1.3^{a}	40 ±1.38^{b}	73 ±0.5^{b}	70 ±0.81^{b}
100	20 ±0.7^{a}	40 ±0.21^{b}	69 ±0.4^{c}	69 ± 0.8^{b}
150	19 ±0.1^{a}	38 ±0.4^{c}	62 ±1.8^{d}	60± 0.4^{c}
200	18 ±1.4^{a}	37 ±0.6^{c}	45 ±0.9^{e}	44 ±0.21^{d}
250	20 ±1.9^{a}	34 ±0.7^{d}	42 ±0.8^{f}	40 ±0.72^{e}
300	20 ±0.7^{a}	33 ±0.9^{d}	37 ±0.17^{g}	35± 0.6^{f}

* Mean ± S.E. values of three separate experiments with six replicates. Values in the same column with the same superscript do not differ significantly ($P \leq 0.05$) according to Duncan's Multiple Range Test.

At 250 mM NaCl the callus showed a 51.2% reduction in growth at the end of 4th week compared to the control while at 300mM NaCl it showed 58.6% of growth inhibition. The concentration of NaCl at which the callus showed 50% reduction in growth compared to the control was taken as the LD_{50} value for all further studies. The callus under non-stressed conditions was loose and creamy in colour while that grown under NaCl was slightly compact and pale yellow.

3. Regeneration studies

a) Regeneration through organogenesis

Among two media tried for regeneration M.S. medium was found superior to L.S. medium (Table 5). To find out the optimum level of hormones for regeneration M.S. medium was supplemented with different levels of NAA, IBA, KN, and BAP and the response was monitored (Table 6). Maximum percentage of regeneration both in control and adapted callus was obtained under the influence of a combination of IBA (1.5 mg/l) and KN (0.5 mg/l). In this combination the control callus showed a regeneration percentage of 68.4 and adapted callus showed regeneration percentage of 28.3. The other combinations tried were IAA + BAP (1.5mg/l + 0.5 mg/l), NAA + KN (1.5 mg/l + 0.5 mg/l). Better promotion of regeneration was observed under a combination of IBA and KN compared to other combinations. These hormones individually failed to show any marked effect on regeneration compared to when tried in combinations. In NaCl adapted calli the percentage of regeneration was low as compared to that of the control. It was noted that a combination of auxin and cytokinin

Table 5: Effect of LS basal media with different growth regulators in percentage of regeneration in rice callus. Data recorded aftger 35th day of inoculation.

Basal media	Growth regulator used (mg/l)	% of callus showing regeneration	No. of plantlets/callus	Length of the plantlets (cm)
LS (Control callus)	0	00.0±0.0	00.0±0.0	00.0±0.0
	NAA (0.25)	00.0±0.00	00.0±0.0	00.0±0.0
	IBA (0.25)	00.0±0.00	00.0±0.0	00.0±0.0
	BAP (0.25)	8.2±0.00	2.3±0.7	4.1±0.9
	IBA+KN (1.5+0.5)	4.7±1.3	1.5±0.1	3.8±0.2
	IAA+BAP (1.5+0.5)	00.0±0.00	00.0±0.0	00.0±0.00
	NAA+KN (1.5+0.5)	11.3±0.1	2.4±1.8	4.1±0.5
LS (Adapted callus)	0	00.0±0.0	00.0±0.0	00.0±0.0
	NAA (0.25)	00.0±0.00	00.0±0.00	00.0±0.0
	IBA (0.25)	00.0±0.0	00.0±0.00	00.0±0.0
	BAP (0.25)	2.1±1.4	1.5±0.7	2.1±0.4
	IBA+KN (1.5+0.5)	00.0±0.00	00.0±0.00	00.0±0.0
	IAA+BAP (1.5+0.5)	00.0±0.00	00.0±0.00	00.0±0.0
	NAA+KN (1.5+0.5)	8.4±0.08	2.4±0.1	2.5±0.3

* Mean ± S.E. values of three separate experiments of four replicates

Plate 3 Different stages of regeneration in rice

a) Control callus

b) Adapted callus

c) Control callus showing regeneration in M.S. + IBA (1.5 mg/l) + KN (0.5 mg/l)

Bar = 1 cm

Plate 3

Table 6 : Standardization of regeneration on M. S basal media with different growth regulators in rice. Data recorded after 35 th day of inoculation.

Basal media	Growth regulators used (mg/l)	% of callus showing regeneration	No. of plant-lets/ callus	Length of the plantlets (cm)
MS (Control callus)	-	00.0±0.0	00.0±0.0	00.0±0.0
	NAA (0.25)	* 34.3 ±1.0	5.1 ±1.3	5.2 ±0.43
	IBA (0.25)	28.71 ±1.2	2.8 ±1.81	5.4 ±0.7
	BAP (0.25)	32.64 ±0.9	4.4 ±2.1	5.8 ± 0.7
	IBA+KN (1.5+0.5)	68.4 ±1.2	8.7 ±0.94	6.21 ± 0.81
	IAA+BAP (1.5+0.5)	27.1 ±1.9	2.7 ±0.8	4.8 ±0.82
	NAA+KN (1.5+0.5)	25 ±0.3	2.5 ±0.71	4.1± 0.5
MS (Adapted callus)	-	00.0±0.0	00.0±0.0	00.0±0.0
	NAA (0.25)	12.3± 0.23	1.2 ±0.4	3.81 ±1.34
	IBA (0.25)	15.2 ±0.21	2.1 ±0.52	3.48 ±0.53
	BAP (0.25)	15.8 ±1.5	2.1 ±0.4	5.21 ±0.42
	IBA+KN (1.5+0.5)	28.34 ±1.7	2.89± 0.82	5.84± 0.21
	IAA+BAP (1.5+0.5)	17.4 ±1.8	2.24 ±1.34	3.81 ±0.25
	NAA+KN (1.5+0.5)	24.2 ±2.7	2.5 ±1.84	3.47± 0.3

* Mean ± S.E. values of three separate experiments with four replicates

significantly improved the regeneration percentage of adapted callus (Table 6). The medium containing IBA and KN produced an average of 8.7 plants per callus in the case of control while the adapted callus showed only 3 plants per callus on 35th day. Regenerants from NaCl adapted callus exhibited a reduction in the

Plate 4

a) Non-embryogenic callus growing on L S. medium + 1 25 mg/l of 2,4-D

b) Embryogenic callus growing on L.S medium + 0 05 mg/l of 2,4-D

Bar = 1 cm

Plate 4

Plate 5 Effect of different concentrations of 2,4-D on rhizogenesis

a) Non-adapted embryogenic callus on L S basal medium

b) Adapted embryogenic callus on L S. basal medium

c) Adapted embryogenic callus showing extensive rooting in 1 25 mg/l of 2,4-D

Bar = 1 cm

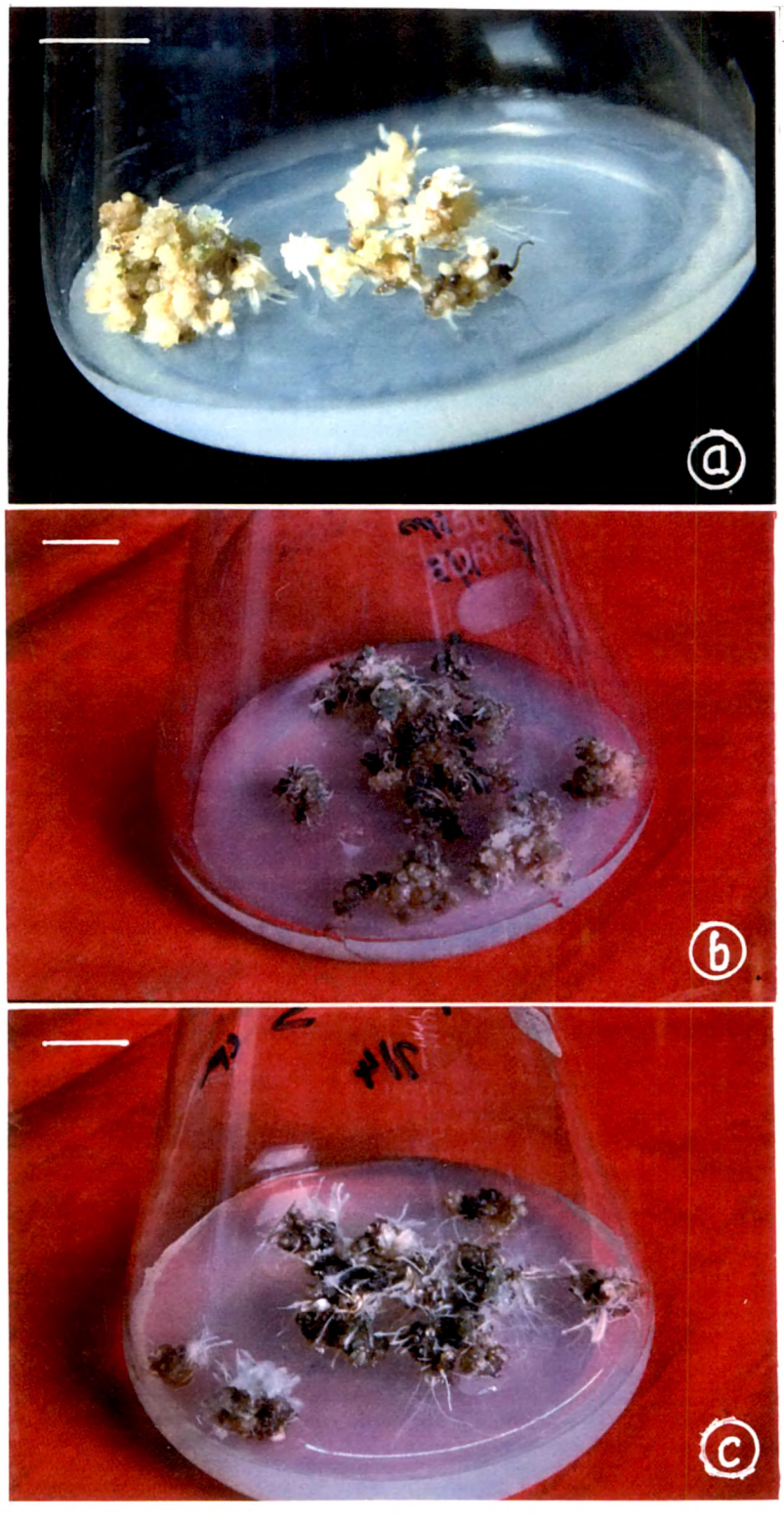


Plate 5

number of roots (Plate 3a, b, c, d). The regenerants were transferred to pots after acclimatization.

4) Somatic Embryogenesis and Production of salt tolerant Embryoids

a) Initiation of Friable Embryogenic callus

Seed derived callus was subcultured on L.S. medium containing 2.5 mg/l of 2,4-D for 40-45 days for the proliferation of the callus. During the subsequent subcultures the level of 2,4-D was brought down to 50, 25 and 0% maintaining the same level of salt (LD_{50}). Reduction in the level of 2,4-D resulted in the production of loose, friable callus with many green spots which is termed as embryogenic callus (Plate 4b). At high concentrations of 2,4-D the callus formed was not friable but was compact, pale yellow termed as non-embryogenic callus (Plate 4a). Rhizogenesis in callus was another problem and this could be reduced to a very low percent by reducing the level of 2,4-D to 0.05 mg/l. Total omission of 2,4-D from the medium resulted in the production of callus free from rhizogenesis (Table 7). Morphology of the callus also differed in case of adapted and non adapted embryogenic cultures. The adapted embryogenic callus showed browning and produced extensive roots on storage and lost its embryogenic potential on prolonged storage (Plate 6c). Frequent subculture (once in 20 days) of embryogenic culture was very important to maintain the embryogenic potential. The level of 2,4-D was found to be very critical in the production of embryogenic, non-embryogenic and rhizogenic cultures. Plate 5b, 5c and 6b shows different morphology of adapted callus under the influence of varying levels of 2,4-D.

Plate 6

a) Non-adapted callus growing on L S. + 2 5 mg/l of 2,4-D Note the extensive rhizogenesis (arrow)

b) Adapted callus growing on L.S. + 2.5 mg/l of 2,4-D

c) Adapted callus showing excessive rooting and browning (arrow) after 3 months in 4 mg/l of 2,4-D

Bar = 1 cm

Plate 6

Table 7 : Effect of different concentrations of 2,4-D on percentage of rhizogenesis during subculture period in rice

LS ± Concentration of 2,4-D used (mg/l)	% of cultures showing rhizogenesis
0.0	*00.0±0.00
0.05	2.6 ±0.2
0.1	14 .8±0.28
1.0	78.5 ±0.52
2.0	100.0 ±0.00
4.0	100.0±0.00

*Mean ± S.E. values of three separate experiments with four replicates

b) Initiation of suspension cultures

A known amount of (# 250 mg) of friable embryogenic callus was inoculated in liquid L.S. medium without any growth regulator and was incubated for 20 days. A uniform cell suspension was obtained by filtering the suspension through stainless steel mesh and this mostly contained pale yellow to white small cell aggregates.

c) Induction of somatic embryogenesis

Induction of somatic embryogenesis was achieved in L.S. medium supplemented with different growth regulators. Initially the suspensions contained cell aggregates of varying morphology (Plate 7, a-d). Frequent observations of these suspensions under stereomicroscope revealed that initially the suspensions

Plate 7 Morphogenetic difference in the cell clumps obtained in embryogenic medium

a) Embryogenic callus with globular embryoids at the initial stage (arrow)

b) Friable callus

c) Nodular callus (arrow)

d) Rhizogenic callus (arrow shows emergence of initial roots)

Bar = 0.01 cm

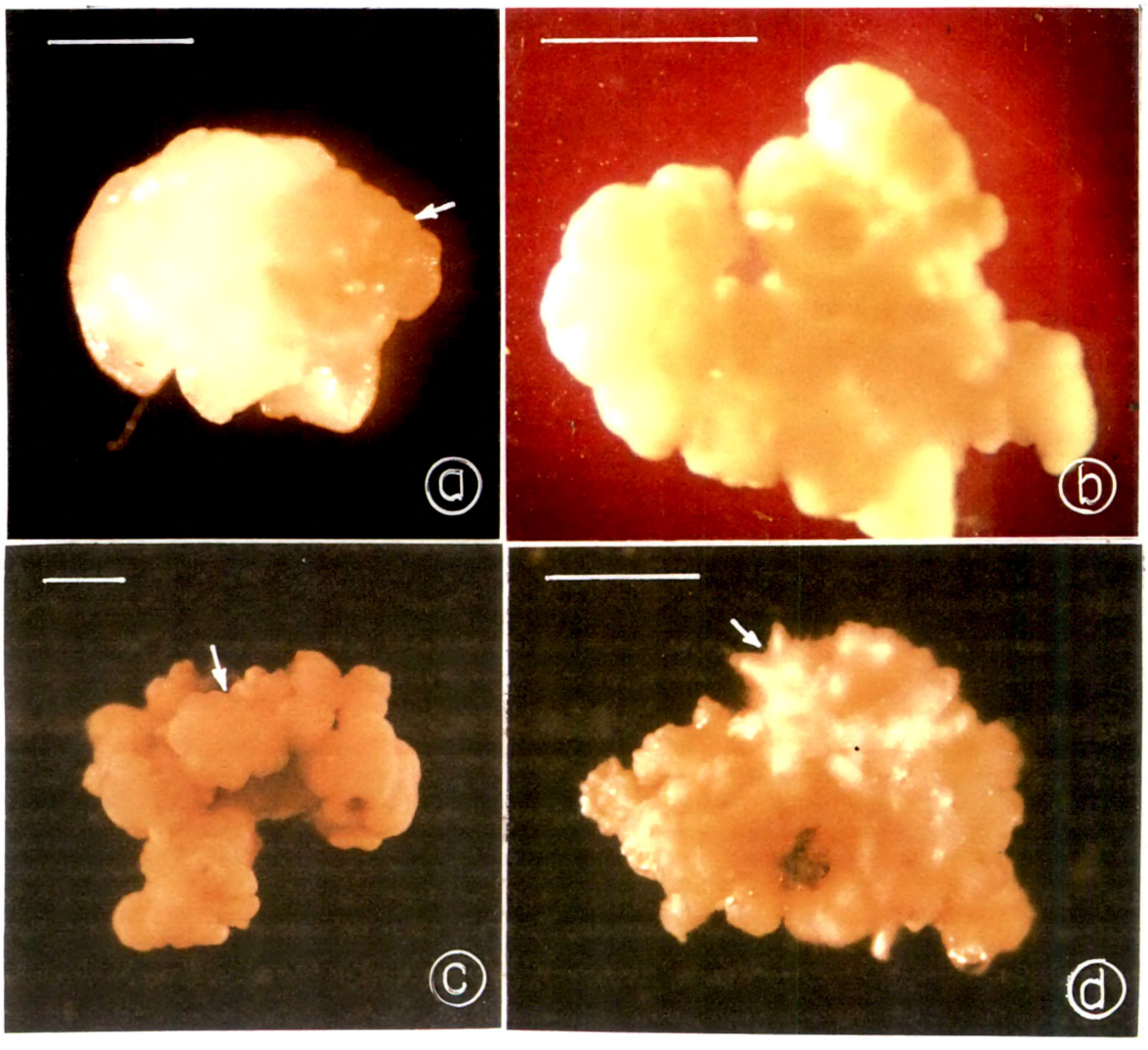


Plate 7

were consisted of highly vacuolated cells, but after 3-4 days they became spherical to oval in shape later forming small group of cells transforming to pro-embryo like structures. A combination of IAA (0.1 mg/l) and BAP (0.5 mg/l) was optimum for inducing maximum number of embryogenic clumps per culture (Table 8). These pro-embryoids became oval-heart shape by 35 days of incubation (Plate 8a and b). Medium without any hormone also could induce somatic embryogenesis in 16% of the cultures while a medium with only auxin failed to induce embryogenesis. Incorporation of IAA or NAA into the medium led to the formation of small calli with occassional rooting. But addition of BAP (0.1 mg/l) to the medium brought about embryogenesis. However incorporation of 2ip failed to induce embryogenesis. However, a combination of 2ip (0.1 mg/l) and BAP (0.5 mg/l) could induce embryogenesis. Thus the combination of IAA (0.1 mg/l) and BAP (0.5 mg/l) was selected for induction of embryoids for all further studies.

Plate 8 a) Isolated globular embryoids obtained in LS+ IAA(0.1 mg/l) and BAP (0.5 mg/l) after 35 days of incubation

b) A single heart shaped embryo with a slight notch (arrow)

Bar = 0.01 cm

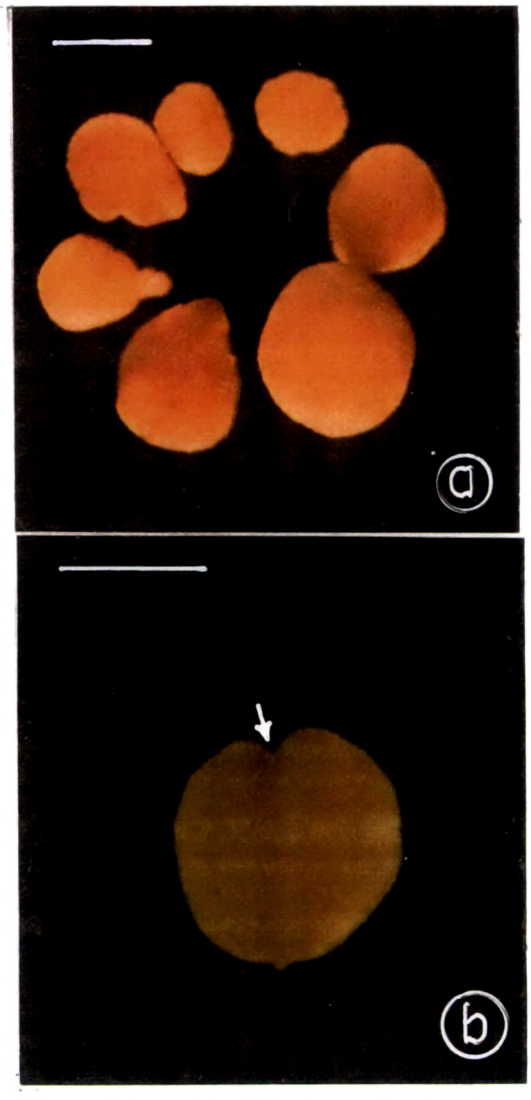


Plate 8

Table 8 : Effect of different concentrations of growth regulators (PGR's) on induction of somatic embryogenesis in rice. Data recorded after 35th day of inoculation

Growth regulators used (mg/l)				% of cultures showing embryogenesis	Mean no. of embryogenic clumps/ culture	Morphology
IAA	BAP	NAA	2 ip			
0.0	0.0	0.0	0.0	*16 ±0.81	4.6 ±1.1	3-4 small globular notch shaped clumps, yellowish organised structures
0.1	0.0	0.0	0.0	-	-	small callus clumps, no organised structures
0.5	0.0	0.0	0.0	-	-	small callus clumps, no organised structures
1.0	0.0	0.0	0.0	-	-	callus clumps, yellowish occasional rooting
2.0	0.0	0.0	0.0	-	-	small callus clumps cells are not dispersed, no organised structures
0.0	0.1	0.0	0.0	12.1 ±0.14	3.8 ±0.02	3-4 globular. Notch shaped structures, slight yellow
0.0	0.5	0.0	0.0	8.31 ±0.06	1.2 ±0.00	globular clumps. organised structures
0.0	1.0	0.0	0.0	8.4 ±0.08	1.00 ±0.00	globular clumps, slight rooting
0.0	2.0	0.0	0.0	10.2 ±1.5	12.5 ±0.24	10-13 globular clumps, slight fusiform, rooting frrom the clump
0.0	0.0	0.1	0.0	-	-	small callus clumps, no organised structures
0.0	0.0	0.5	0.0	-	-	small callus clumps, rooting from the clumps
0.0	0.0	1.0	0.0	-	-	no organised structures, small callus clumps produced roots only
0.0	0.0	2.0	0.0	-	-	yellowish callus clumps, extensive rooting from the clumps

Table 8 contd-

Growth regulators used (mg/l)				% of cultures showing embryoge-nesis	Mean no. of embryogenic clumps/ culture	Morphology
IAA	BAP	NAA	2 ip			
0.0	0.0	0.0	0.1	-	-	unorganised structures with only rooting
0.0	0.0	0.0	0.5	-	-	small callus clumps without any morphology of embryo
0.0	0.0	0.0	1.0	-	-	unorganised structures only rooting
0.0	0.0	0.0	2.0	-	-	unorganised structures only rooting
0.1	0.5	0.0	0.0	46.2 ±0.5	22.0 ±0.09	typical organised structures clumps showed rooting, good response to germination
0.5	0.1	0.0	0.0	24.3 ±0.15	15.4 ±0.8	small organised embryogenic clumps. some of the clumps showed rooting
1.0	2.0	0.0	0.0	28.4 ±0.2	16.5 ±0.7	small organised embryogenic clumps, yellowish in colour
2.0	1.0	0.0	0.0	26.71 ±0.7	15.58 ±1.84	embryogenic clumps, green to yellow in colour slight rooting from the clumps
0.0	0.0	0.1	0.5	21.32 ±0.43	12.3 ±0.5	Green clumps, showed rooting
0.0	0.0	0.5	0.1	22.74 ±0.5	13.1 ±0.3	Yellowish-green clumps showed rooting from the clumps
0.0	0.0	1.0	2.0	12.25 ±0.72	4.1 ±0.4	small clumps, organised yellowish showed rooting
0.0	0.0	2.0	1.0	14.4 ±1.34	5.8 ±0.82	small clumps, mostly mingled with callus clumps showed rooting

* Mean ± S.E. values of three independant experiments with four replicates.

Plate 9 Embryoids produced from NaCl (LD_{50}) adapted cultures

a) Isolated globular embryoids obtained in LS + IAA (0 1 mg/l) and BAP (0.5 mg/l)

b) A group of four embryoids

c) A single heart shaped embryo

d) Precocious germination of the embryoids in the same medium

Bar = 0 01 cm

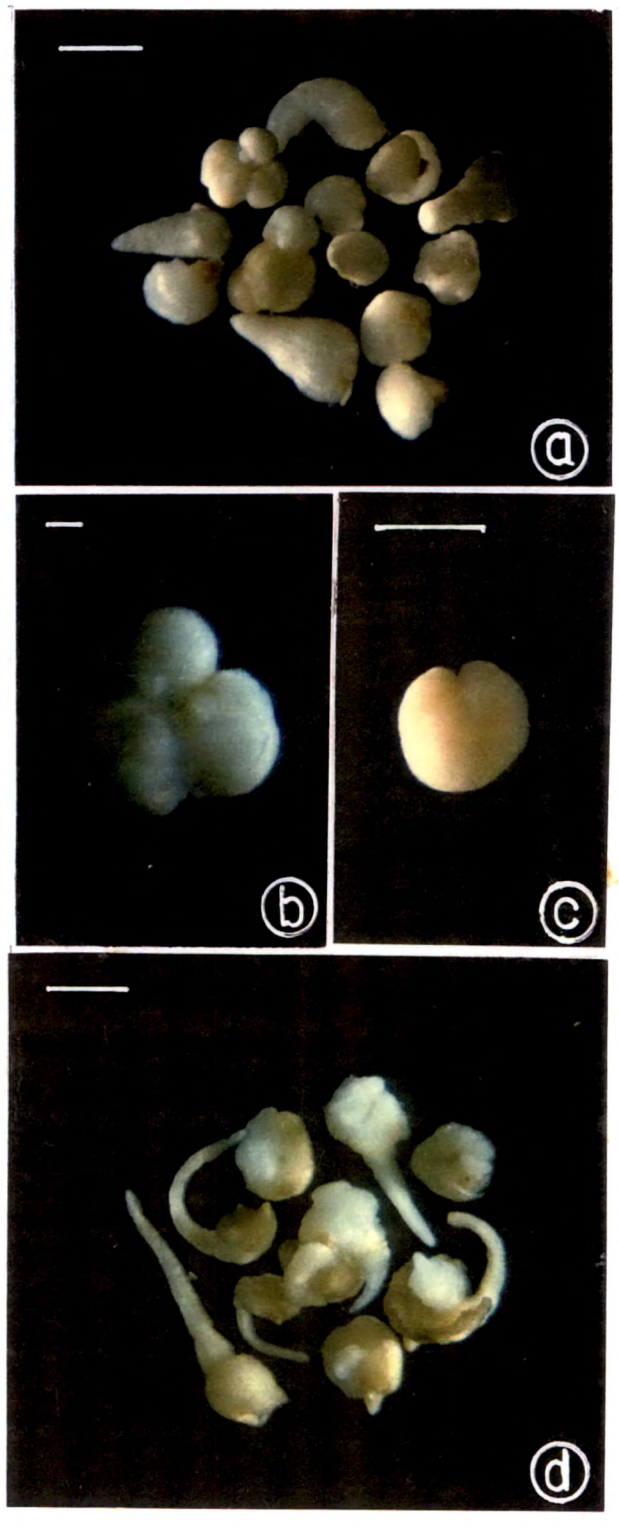


Plate 9

d) Induction of salt tolerant embryoids

Salt tolerant embryoids were isolated from salt adapted (LD_{50} NaCl) and salt exposed cell lines. Embryoids derived from adapted cells showed a reduction in rhizogenesis and improvement in embryogenesis compared to the controls. Production of embryoids was observed in 61.2% of the cultures while non-adapted cultures showed only 48% of embryogenesis (Table 9).

Table 9 : Effect of LD_{50} concentration of NaCl (0.25M) on percentage of embryogenesis in rice. Medium used was LS supplemented with 0.1 mg/l of IAA and 0.5 mg/l of BAP

Days after inoculation	Treat-ments	Total no. of embryogenic clumps/culture	% of cultures showing embryogenesis	Total no. of rhizogenic clumps/culture	% of cultures showing rhizogenesis
10	C	00.0±0.0	00.0±0.0	00.0±0.0	00.0±0.0
	A	00.0±0.0	00.0±0.0	00.0±0.0	00.0±0.0
	NA	00.0±0.0	00.0±0.0	00.0±0.0	00.0±0.0
20	C	15.6 ±1.3	30.4 ±2.1	38.15 ±0.7	68.6±1.3
	A	18.5 ±0.3	35.4 ±2.81	34.2±0.81	63.1±1.8
	NA	17.2 ±0.5	33.81±0.7	35.1±0.72	64.3±2.3
30	C	23.4 ±1.3	44.3±0.8	24.3±0.8	53.0±0.34
	A	29.8 ±2.1	48.4±0.71	21.2±0.7	49.3±2.1
	NA	25.4 ±0.7	45.3±0.21	24.31±0.78	54.3±0.31
40	C	27.4 ±0.8	46.4±0.8	21.0±0.14	50.1±2.8
	A	34.4 ±0.32	61.4±0.74	15.4±0.71	38.3±0.31
	NA	29.3 ±0.78	48.3±0.92	19.4±0.81	47.4±0.48

C - Control, A-Adapted, NA - Nonadapted

* Mean ± S.E. values of three independant experiments with five replicates.

Plate 10 Embryoids produced from non-adapted cultures under LD_{50} concentration of NaCl

a) Globular embryoids obtained in LS + IAA (0.1 mg/l) and BAP (0.5 mg/l)

b) Abnormal germination of embryoids under the influence of NaCl

c) A single embryoid

d) An elongated root produced from an embryogenic clump

e) Abnormal growth and death of the cells under LD_{50} concentration of NaCl

Bar = 0.01 cm

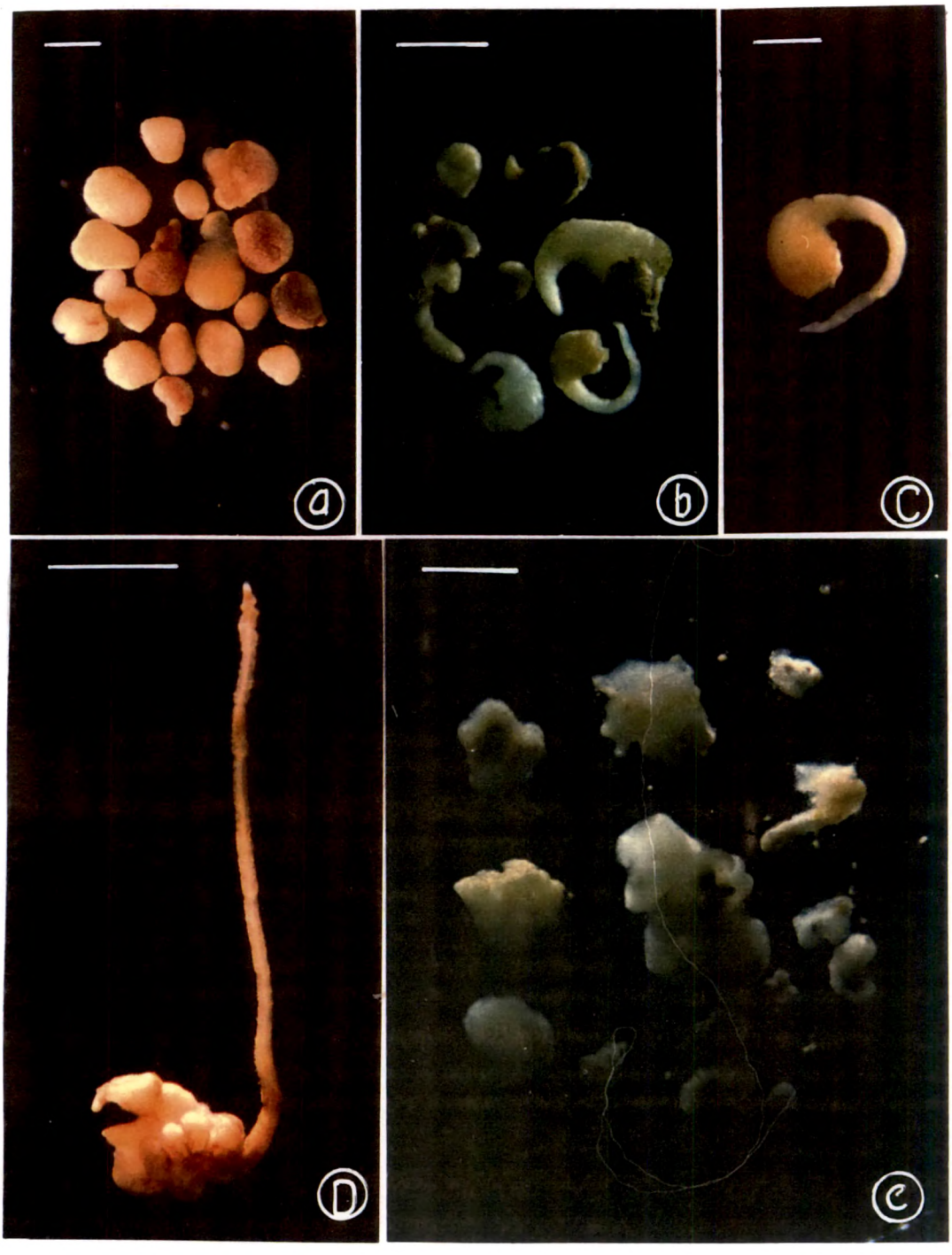


Plate 10

Plate 9, a-d shows isolated embryoids obtained from adapted cell lines. The embryoids derived from adapted cell lines showed a significant reduction in rhizogenesis compared to those formed under other treatments. The embryoids from non adapted cultures showed elongated roots, browning and perished if kept in the same medium for a long period (Plate 10, a-e).

5) Experiments to improve somatic embryogenesis

In order to improve somatic embryogenesis and enhance maturation and germination different manipulations were done in the embryogenic media.

a) Effect of Proline

Incorporation of proline at 2, 4 and 8 mM concentrations has significantly stimulated the percentage of embryogenesis both in control and adapted cultures (Table 10). At 8 mM proline both control and adapted cultures showed a two fold increase in the percentage of cultures with somatic embryogenesis and also the number of somatic embryoids per culture. These embryoids produced under the influence of proline were mostly green, organised with well developed coleoptile, scutellum and showed a good germination (Plate 11, a-d).

b) Effect of hydroxyproline

Addition of hydroxyproline to the medium at 4 and 8 mM concentrations improved the percentage of embryogenesis as well as the number of embryoids in adapted cultures (Table 11). Among the concentrations tried better response in terms of embryogenesis and number of embryoids was observed under the influence of 4 mM concentration of hydroxyproline.

Plate 11 Effect of proline on embryogenesis in rice

a) Green and healthy embryoids produced under the influence of 8mM of proline

b&c) Adapted embryoids showing good germination under the influence of 8mM of proline

d) Adapted embryoids showing good germination with many green embryogenic clumps under the influence of 8mM proline - an enlarged view

Bar = 1 cm

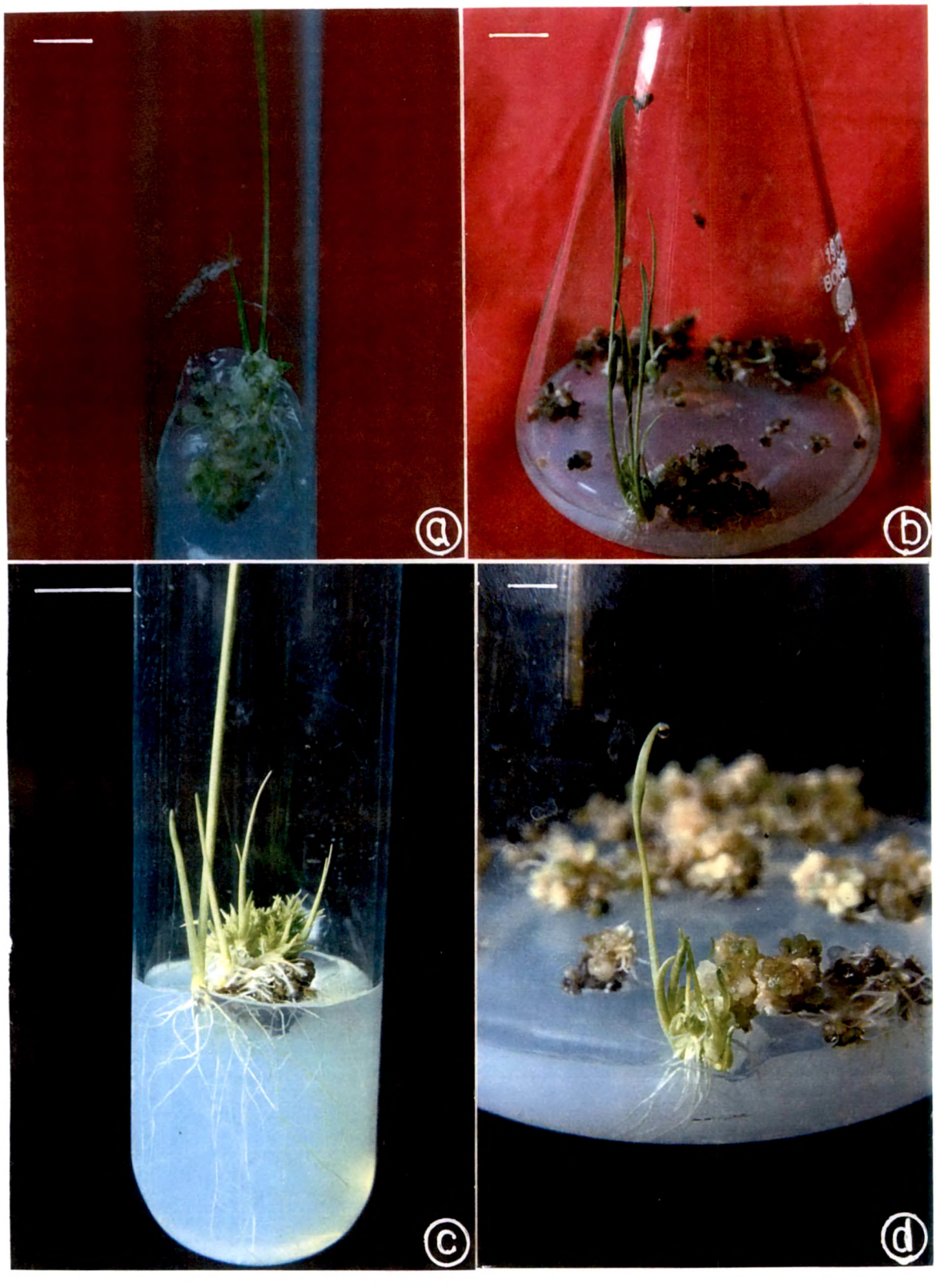


Plate 11

Table 10 : Effect of different concentrations of Proline (0-32mM) on somatic embryogenesis in rice . Medium used was LS supplemented with IAA (0.1 mg/l) and BAP (0.5 mg/l). Data recorded after 35th day of inoculation

Concentration of Proline used (mM)	Treatments	% of cultures showing embryogenesis	Mean no. of somatic embryos/culture
0.0	C	*46.4 ±0.5	22.4 ±0.30
	A	32.12±0.2	13.8 ±0.21
2.0	C	48.25 ±0.7	27.3 ±0.4
	A	34.2 ±1.81	15.3 ±1.5
4.0	C	51.6 ±0.5	28.1 ±0.8
	A	47.9 ±2.41	22.4 ±1.8
8.0	C	76.7 ±0.4	49.8 ±1.3
	A	71.8 ±0.7	47.9 ±0.7
16	C	27.7 ±0.2	9.1 ±0.8
	A	25.4 ±0.8	8.3 ±0.6
32	C	21.4 ±0.3	6.39 ±0.3
	A	12.3 ±0.4	3.8 ±0.5

C - Control, A-Adapted

* Mean ±S.E. values of three independant experiments with four replicates

Table - 11: Effects of different concentrations of hydroxyproline on somatic embryogenesis in rice. Medium used was LS supplemented with IAA (0.1 mg/l) and BAP (0.5 mg/l). Data recorded after 35th day of inoculation

Concentration of H. Proline used (mM)	Treatments	% of cultures showing embryogenesis	Mean no. of somatic embryos/culture
0.0	C	*46.4 ±0.5	22.4 ±0.30
	A	32.1 ±0.2	13.8 ±0.2
2.0	C	12.0 ±0.8	4.2 ±0.7
	A	22.4 ±0.3	5.6 ±0.5
4.0	C	16.4 ±0.2	4.8 ±1.3
	A	28.4 ±0.4	10.2±1.2
8.0	C	12.4 ±0.3	3.9 ±0.32
	A	16.8 ±0.4	5.1 ±0.4
16	C	8.4 ±0.4	2.8 ±0.4
	A	10.3 ±0.3	3.3 ±0.3
32	C	5.2 ±0.4	1.38 ±0.2
	A	7.4 ±0.8	2.1 ±0.1

C - Control, A- Adapted

* Mean ±S.E. values of three independent experiment with four replicates.

(c) Effect of Polyamine

(i) Effect of Putrescine

Putrescine (0-0.8 mM) was incorporated into the medium to examine its effect on embryogenesis. Among different levels of putrescine tried 0.1 mM brought about 20 and 23% of embryogenesis in control and adapted cultures respectively (Table 12).

Plate 12 a) Embryoids undergoing extensive rhizogenesis under varying concentrations of putrescine (0-0 8mM)

b) Embryogenic clump showing heavy rooting under 0 8mM of putrescine

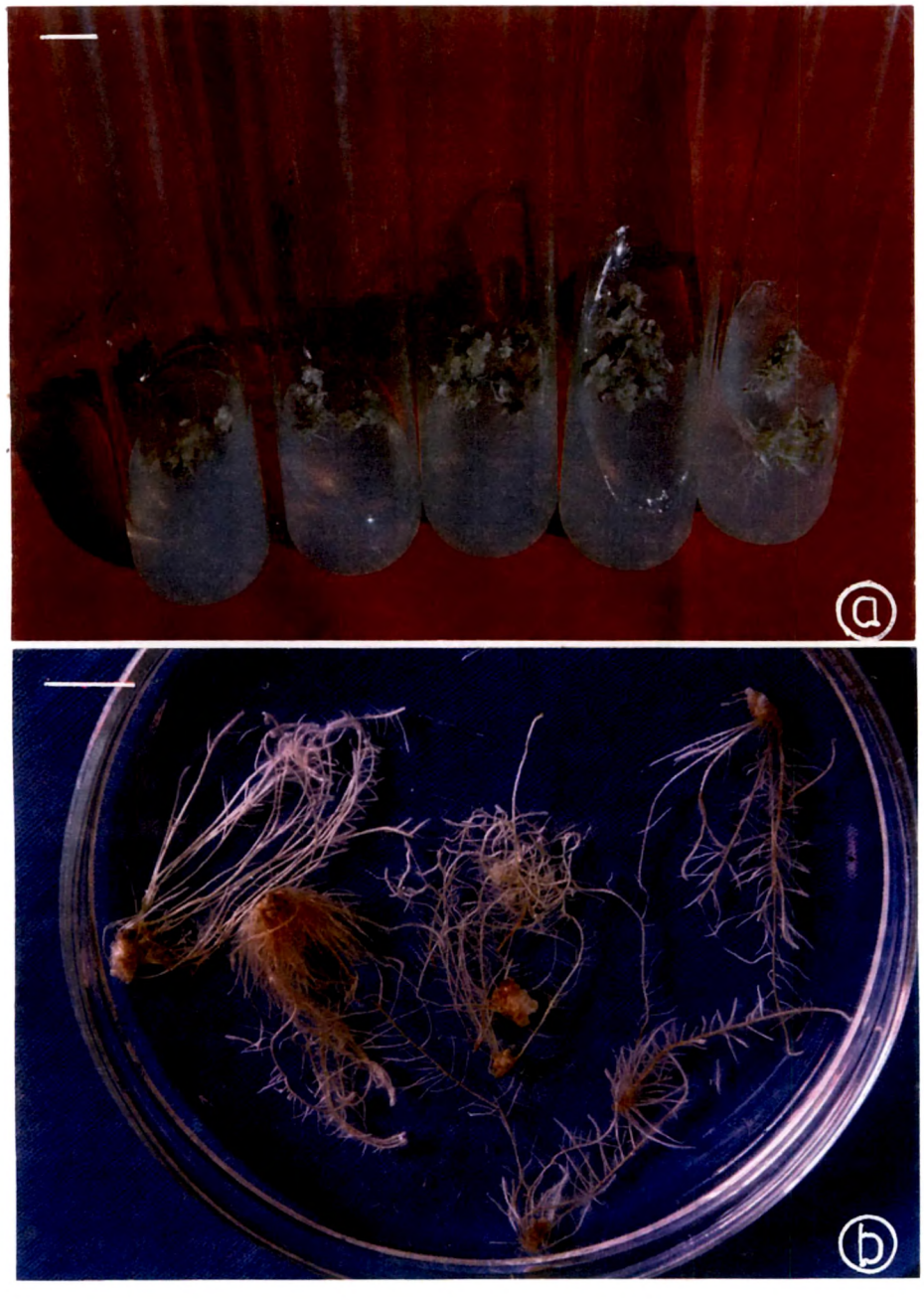


Plate 12

Plate 13 Effect of putrescine on rhizogenesis in adapted embryoids

a) Control

b) Under 0 4mM of putrescine

c) Under 0.8mM of putrescine

Bar = 1 cm

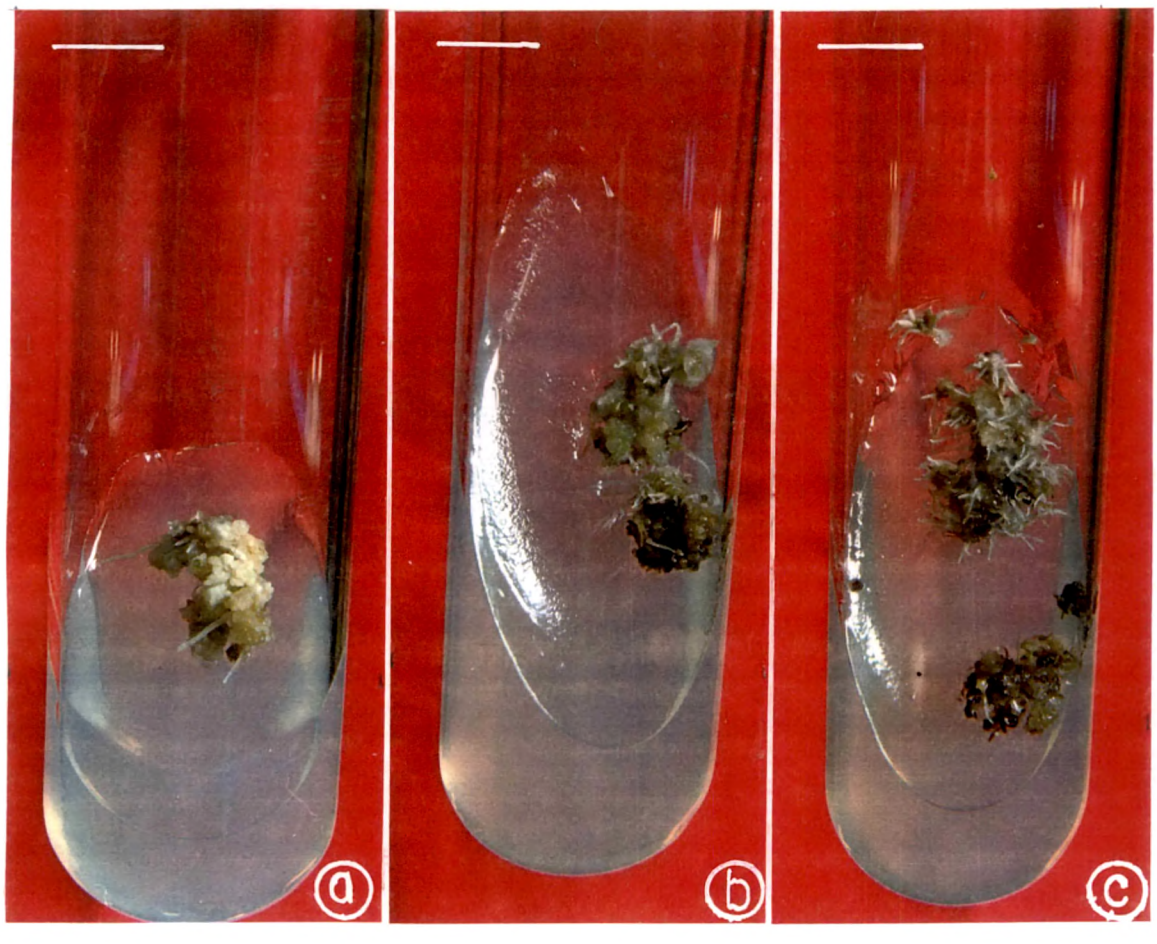


Plate 13

Table 12: Effect of different concentrations of Putrescine (0-0.8mM) on somatic embryogenesis in rice. Medium used was L.S. supplemented with IAA (0.1 mg/l) and BAP (0.5 mg/l). Data recorded after 35th day of inoculation.

Concentration of Putrescine used (mM)	Treat-ments	% of cultures showing rhizogens	Mean no. of rhizogenic clumps/ culture	% of cultures showing embryo-genesis	Morphology
0.0	C	*00.0±0.0	00.0±0.0	46.4±0.5	small clumps, slightly elongated with rooting
0.0	A	00.0±0.0	00.0±0.0	32.12±0.2	4-5 small clumps, slight browning
0.1	C	73.4±0.1	43.1±0.71	20.4±0.7	whitish pale green, less rooting
0.1	A	75.8±0.8	44.2±0.98	23.1±0.81	green spot with less roots
0.2	C	94.1±0.2	48.1±0.6	10.4±0.82	green spot with many roots
0.2	A	87.4±0.2	45.8±0.5	12.8±0.91	many clumps with roots
0.4	C	100.0±0.1	52.4±1.3	00.0±0.0	clumps produced extensive rooting
0.4	A	100.0±0.24	54.1±3.1	00.0±0.0	extensive rooting yellowish
0.8	C	100.0±0.1	52.8±0.7	00.0±0.0	Extensive rooting, yellow clumps
0.8	A	100.0±0.4	55.4±0.8	00.0±0.0	highly rooting clumps

C-Control, A-Adapted

* Mean ± S.E. values of three separate experiments with three replicates.

It was interesting to note that with increase in concentration of putrescine there was a parallel increase in rhizogenesis (Plate 12 a and b). Under putrescine treatment the adapted cultures showed a reduced rhizogensis (Plate 13,a-c). Prolonged

Plate 14 a) Non-adapted embryoids showing germination under 0 5 mM concentration of MGBG

b) Adapted embryoids showing germination under 0.5mM concentration of MGBG

Bar = 1 cm

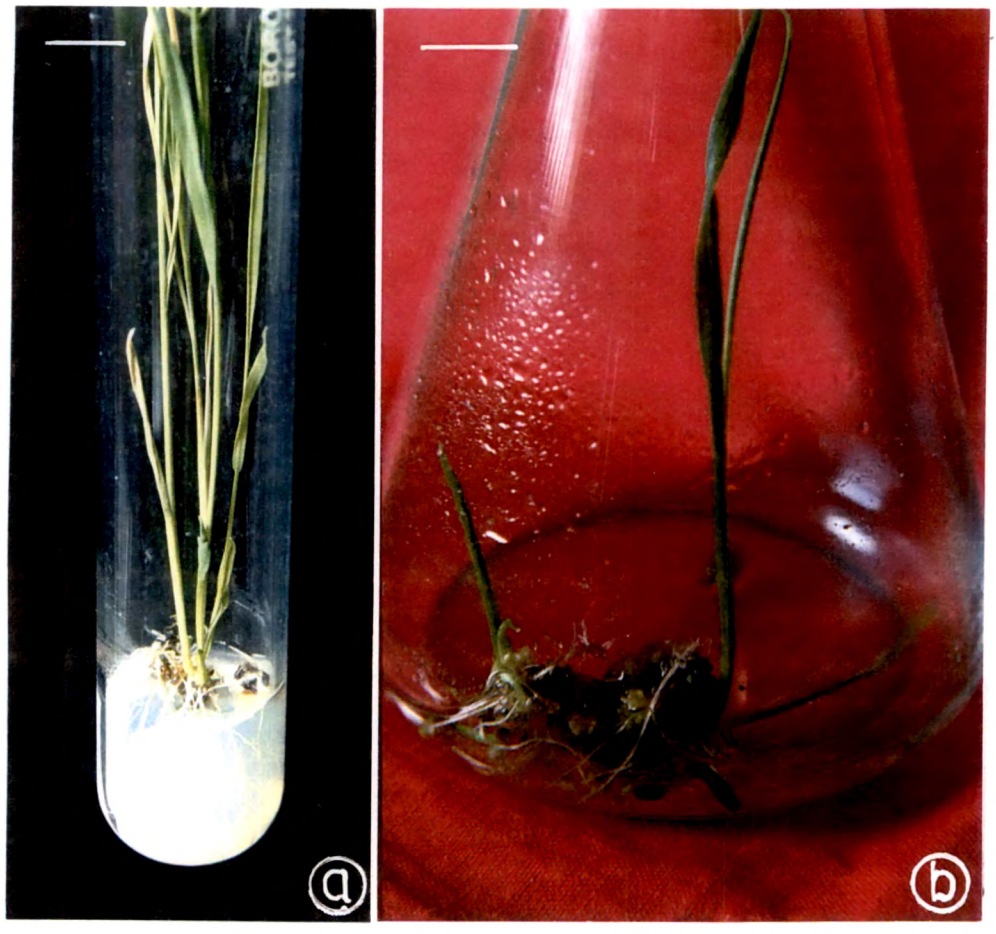


Plate 14

incubation of embryoids in the putrescine containing medium resulted in inhibition of the growth of the plantlets.

The response of embryogenic cultures in presence of MGBG was just the opposite of that observed under the influence putrescine. MGBG at different concentrations promoted embryogenesis and germination of embryoids (Table 13). The embryoids gave rise to healthy green plantlets (Plate 14, a and b). MGBG reduced rhizogenesis to a great extent in the embryoids.

Table 13: Effect of different concentrations of MGBG on percentage of embryogenesis in rice. Data recorded after 45th day of incubation.

Concentration of MGBG used (mM)	Treatments	% of cultures showing embryogenesis	Average no. of plantlets / culture
0.0	C	*46.4±0.5	4.1±0.1
0.0	A	32.1±0.2	3.2±0.7
0.25	C	41.3±0.8	3.9±0.7
0.25	A	28.1±0.4	3.8±0.3
0.5	C	58.3±0.7	5.1±0.1
0.5	A	37.1±0.6	4.3±0.2
1.0	C	38.7±0.1	2.4±0.7
1.0	A	27.4±0.2	2.0±0.1

C = Control, A = Adapted

* Mean ± S.E. values of three separate experiments with three replicates.

6) Experiments to control the problem of rhizogenesis

The main problem faced during the period of maturation and germination of somatic embryos was precocious rhizogenesis. Premature development of roots

Plate 15 Varying morphology of embryogenic clumps obtained in suspension cultures from non-adapted cultres

a) Extensive rhizogenesis of embryogenic cell aggregates in L.S medium supplemented with 0.1 mg/l IAA + 0 5 mg/l of BAP

b) Slightly reduced rhizogenesis in embryogenic cell aggregates in above medium with 100mM NaCl

c) Reduced rhizogenesis in embryogenic cell aggregates in same medium with 50 mM NaCl

Bar = 0 01 cm

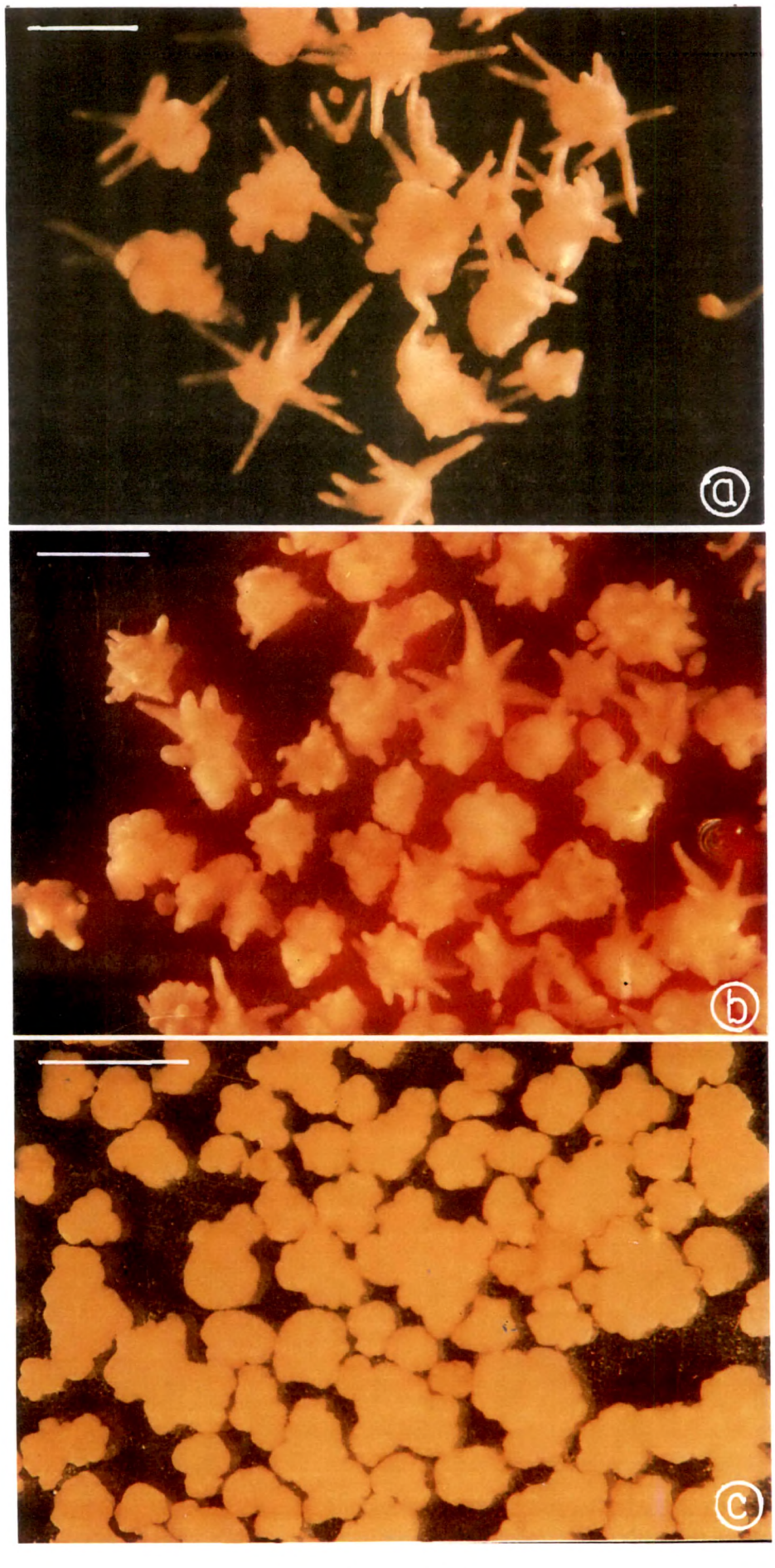


Plate 15

obstructed the further development of embryoids. The root induction was much faster than the shoot induction.

a) Influence of NaCl

It was possible to completely check the premature rhizogenesis in embryoids from controlled cell lines by maintaining them in the embryogenic medium containing 50 mM NaCl (Plate 15,a-c). With an increase in the concentration of NaCl there was a parallel increase of rhizogenesis (Table 14).

Table 14 : Effect of different concentrations of NaCl on reducing rhizogenesis on embryogenic clump derived from non adapted callus of rice. Data recorded after 35th day of inoculation

Con. of NaCl used (mM)	% of embryogeneic clumps showing rhizogenesis
00.0	*58.4 ±1.54
50	0.00 ±0.00
100	13.4±2.3
150	24.3±0.37
200	37.0±0.32
250	48.7±0.43

* Mean ± S.E. values of three independant experiments with four replicates.

b) Influence of activated Charcoal (AC)

Incorporation of activated charcoal did not totally prevent the rhizogenesis in embryoids. However almost 58% reduction in rhizogenesis could be observed when charcoal was added to the medium at concentration of 8% (Table 15). The

Plate 16 Difference types of isolated embryo like structures obtained in maturation medium

a-c Abnormal embryos

d) A well developed embryoid in LS + 4% sucrose

sc- scutellum

cr-coleorhiza

cl-coleoptile

Bar=0 01 cm

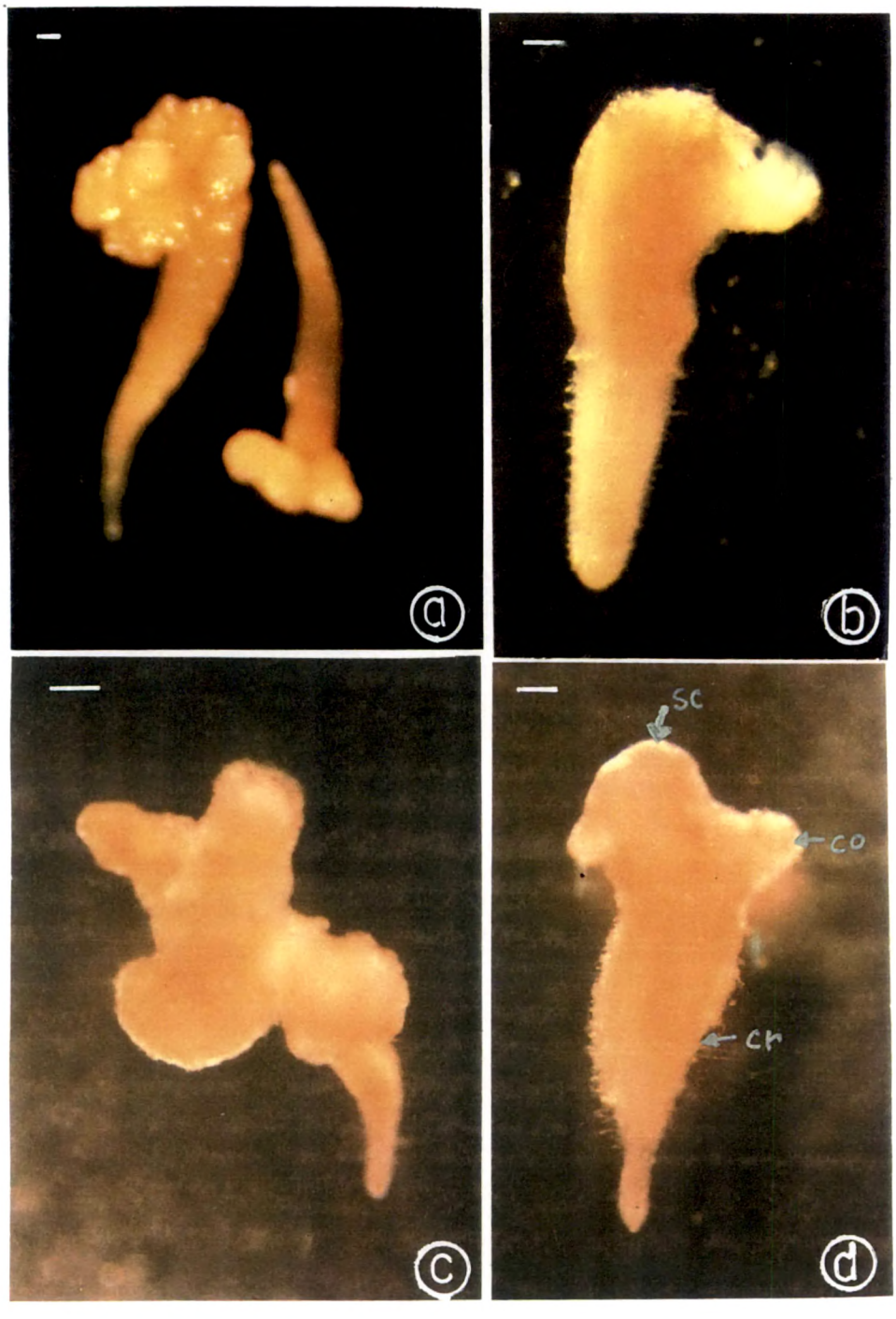
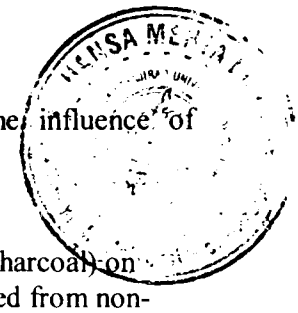


Plate 16



embryoids showed poor maturation and germination under the influence of activated charcoal.

Table 15 : Effect of different concentrations of AC (activated charcoal) on reducing rhizogensis on embryogenic clumps derived from non-adapted callus of rice. Data recorded after 35th day of inoculation

Concentration of AC used (%)	% of embryogeneic clumps showing rhizogenesis
0.0	*58.4±1.54
2.0	61.8±2.3
4.0	52.4±3.1
8.0	42.0±1.3

* Mean ± S.E. values of three separate experiments with four replicates.

7) Maturation studies

The developing embryoids have been transferred to maturation medium containing various osmotica such as sucrose, PEG-6000 and mannitol.

a) Effect of sucrose

Among three levels of sucrose tried (LS + 2, 4, 8%) best result with respect to maturation were observed at 4% (w/v) (Table 16). The matured embryoids exhibited well developed scutellum, coleoptile and root (Plate 16d). Omission of sucrose from the medium resulted in poor maturation of embryoids (Plate 16, a-c).

Table 16: Effects of different concentrations of sucrose on maturation of somatic embryoids in rice

Concentration of sucrose used (%)	Treatments	% of embryogenic clumps showing maturation	Morphology of embryogenic clumps
0.0	C	00.0±0.0	-
0.0	A	00.0±0.0	-
2.0	C	26.3±0.2	Elongated structures, small clumps
2.0	A	15.8±0.34	small clumps, browning, slightly yellowish
4.0	C	100.0±0.23	uniform clumps in culture, well developed scutellum, coleoptile and root
4.0	A	100.0±0.4	organised uniform structures slight browning
8.0	C	57.1±0.31	small clumps, well uniform
8.0	A	52.4±0.36	uniform clumps, organised, browning

* Mean ±S.E. values of three separate experiments with four replicates.

b) Effect of PEG-6000 (Polyethylene Glycol)

Addition of PEG (LS+2% sucrose + 1.5%, 3% or 6%) to the maturation medium failed to induce maturation of embryoids. PEG exposed embryoids showed excessive rooting and browning and failed to germinate (Table 17).

Plate 17 Embryoids under mannitol treatment

a) Control embryoids under 25mM mannitol

b) Adapted embryoids under 25mM mannitol

c&d) Mannitol treated embryoids showing poor germination in non-adapted and adapted embryoids

Bar = 1 cm

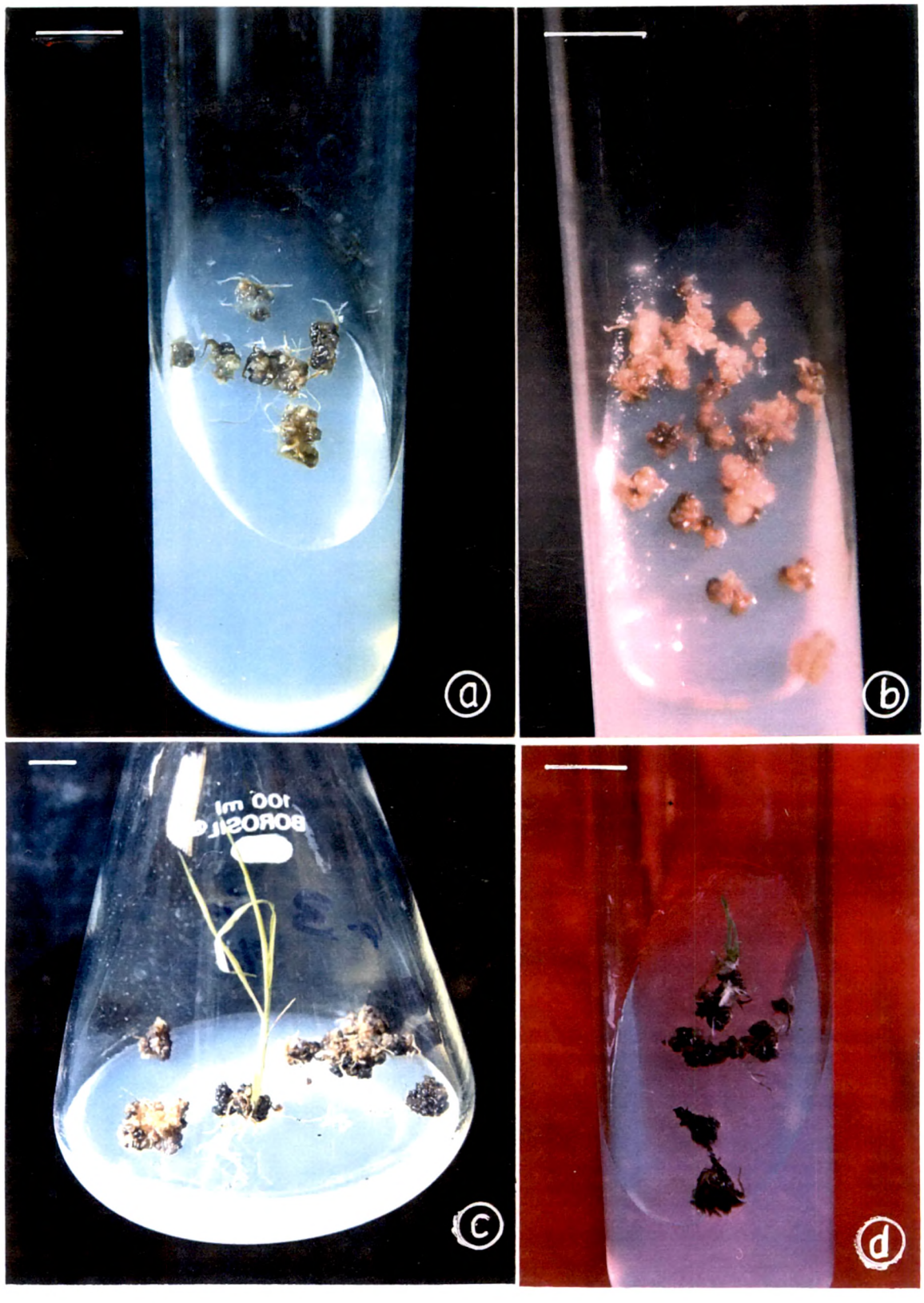


Plate 17

Table 17: Effect of different concentrations of PEG (Polyethylene glycol) on percentage of maturation on somatic embryos in rice

Concentration of PEG used (%)	Treatments	% of embryogenic clumps showing maturation	Morphology of the embryogenic clumps
0.0	C	*26.3±0.2	small clumps slightly elongated with roots
0.0	A	15.8±0.3	small clumps with roots, browning
1.5	C	0.00±0.0	small clumps with roots, browning
1.5	A	0.00±0.0	extensive rooting , dying and browning
3.0	C	0.00±0.0	extensive rooting
3.0	A	0.00±0.0	extensive rooting and browning
6.0	C	0.00±0.0	slight brown embryoids, turned black
6.0	A	0.00±0.0	blackish embryoids, died very fast

C- Control, A-Adapted

* Mean ±S.E. values of three separate experiments with four replicates.

c) Effect of mannitol

The maturation response of embryoids to varying levels of mannitol was tried and it has been observed that mannitol 25 and 50 mM levels marginally improved the maturation of embryoids (Table 18). The results observed under the influence of mannitol was not at all comparable to those obtained under the influence of sucrose. Mannitol treated embryoids showed a poor germination afterwards (Plate 17, a-d).

Plate 18 Embryoids showing precocious germination in the embryogenic medium itself

Bar = 0 01 cm

Plate 18

Table 18: Effects of different concentrations of mannitol (mM) on percentage of maturation of somatic embryos in rice

Concentration of mannitol used (mM)	Treatments	% embryogenic clumps showing maturation	Morphology of the embryogenic clumps
0.0	C	*26.3±0.2	small clumps, slightly elongated
0.0	A	15.8±0.3	slightly browning, no response to germination
25	C	22.4±0.89	small group of embryogenic clumps, slight uniform
25	A	16.3±0.4	organised structures yellowish
50	C	12.4±0.7	slightly organised clumps
50	A	18.3±0.31	small clump, mostly uniform, slightly browning
100	C	13.1±0.48	organised, yellow to dark structures
100	A	14.3±0.71	slightly browning, mostly uniform

C-Control, A-Adapted

* Mean ± S.E. values of three seperate experiments with four replicates.

8) Germination studies

Precocious germination of embryoids in the embyrogenic medium itself was a main problem (Plate 18). Matured embryoids were put for germination in various media. Embryoids when placed on only solidified agar failed to show any sign of germination. But transfer of matured embryoids to LS medium containing varying levels of sucrose slightly improved their germination percentage (Table 19). Highest percentage of germination (20%) was observed at 5% sucrose concentration. Plate 19, a-d shows different stages of germination.

Table 19 : Effect of different concentrations of sucrose on percentage of germination of somatic embryoids in rice

Media + % of sucrose	Treatments	% of germination
water-agar	C	*0.00±0.00
water-agar	A	0.00±0.00
LS + 1.25	C	8.3±0.1
LS + 1.25	A	6.1±0.2
LS + 2.5	C	10.4±0.8
LS + 2.5	A	8.3±0.4
LS + 5	C	12.4±0.1
LS + 5	A	10.8±0.8
1/2LS + 1.25	C	18.1±1.4
1/2 LS + 1.25	A	16.4±0.7
1/2LS + 2.5	C	20.3±0.2
1/2 LS + 2.5	A	15.4±0.3
1/2LS + 5	C	22.3±1.3
1/2 LS + 5	A	18.1±0.7

C- Control, A- Adapted

* Mean ± S.E. values of three experiments with three replicates.

Plate 19 Different stages of germination of embryoids

a) Isolated embryoids showing initial stage of germination (arrow slight emergence of scutellum

b) An embryogenic clump showing initial emergence of shoot and root

R - root

S - shoot

c&d) Further stages of germination

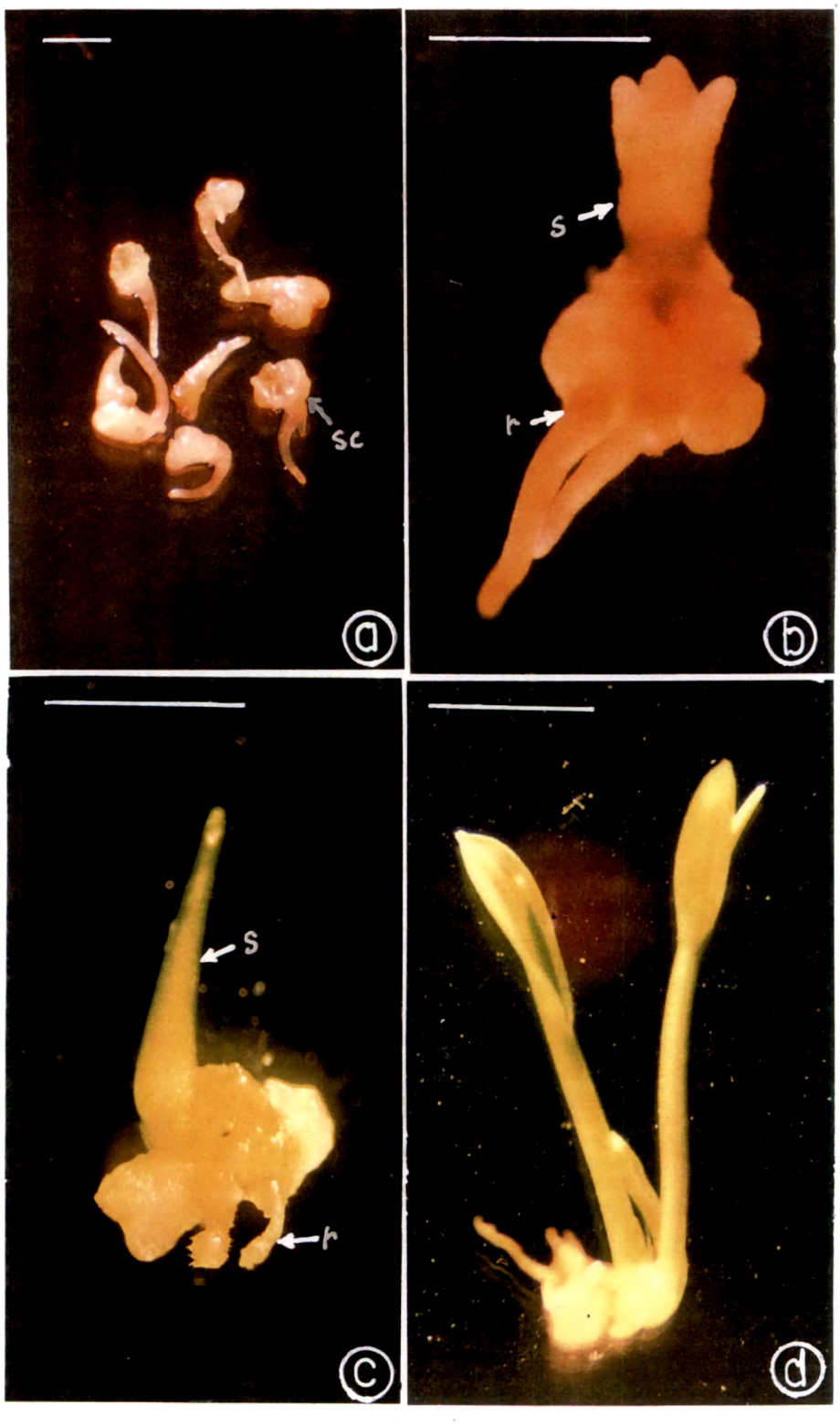


Plate 19

Plate 20

a) Germination of embryoids and fully grown plantlets in 1/2 strength LS medium + 5% sucrose + 8mM proline

b) Adapted embryoids showing poor germination in semi-solid medium

c&d) Control and adapted embryos showing good germination in liquid medium

Bar = 1 cm

Plate 20

Incorporation of proline into the medium resulted in a significant improvement of germination. The embryoids derived from non-adapted and adapted cells showed 72.3% and 64.2% of germination respectively (Table 20).

Table 20: Effect of different concentrations of proline on percentage of germination of somatic embryoids in rice

Media + Growth regulators	Treatments	% of germination
Control	C	*22.3±1.3
Control	A	18.1±0.7
1/2LS+5% Sucrose + 2mM proline	C	25.3±0.71
1/2LS+5% Sucrose + 2mM proline	A	22.4±0.81
1/2LS+5% sucrose+4mM Proline	C	38.1±0.5
1/2LS+5% sucrose+4mM Proline	A	32.1±0.7
1/2LS+5% Sucrose+8mM Proline	C	71.4±1.3
1/2LS+5% Sucrose+8mM Proline	A	66.8±0.8
1/2 LS+5% Sucrose+16 mM Proline	C	24.3±0.2
1/2 LS+5% Sucrose+16 mM Proline	A	21.3±0.7

C - Control, A- Adapted

* Mean ± S.E. values of three separate experiments with four replicates

A comparison of germination behaviour of embryoids on semi-solid and liquid media revealed better performance in the liquid medium (Plate 20,a-d). Embryoids produced 3-4 roots and 2 shoots per clump on solid medium while they produced 12-15 roots and 7-8 shoots in liquid cultures. Plantlets formed from embryoids in liquid medium were more healthy and green compared to those on

Plate 21 a) An adapted embryogenic clump showing albino plant in germination medium

b) Further growth of the albino plant

c) Adapted embryogenic clump showing both albino and green plants

Bar = 1 cm

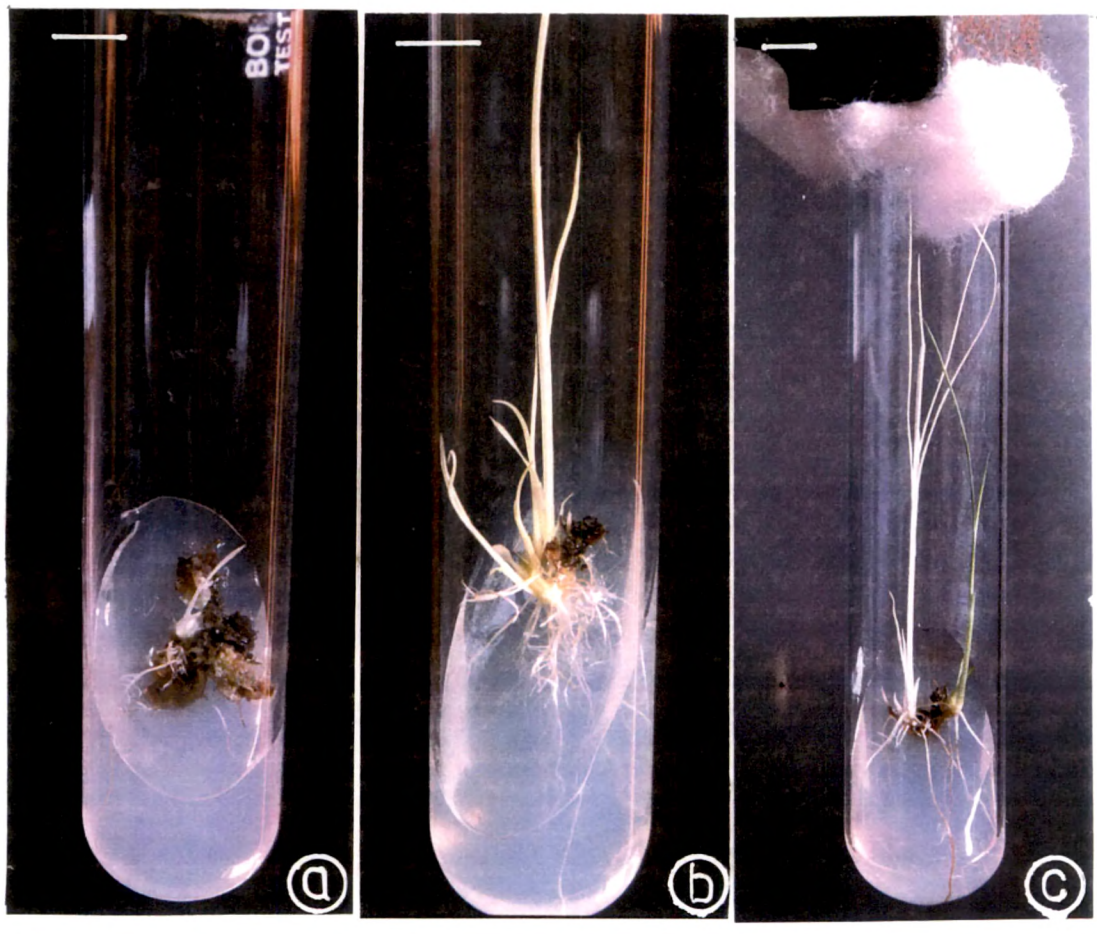


Plate 21

Plate 22 a) Encapsulated somatic embryo clusters of rice

b) Encapsulated somatic embryos in germination medium

Bar = 1 cm

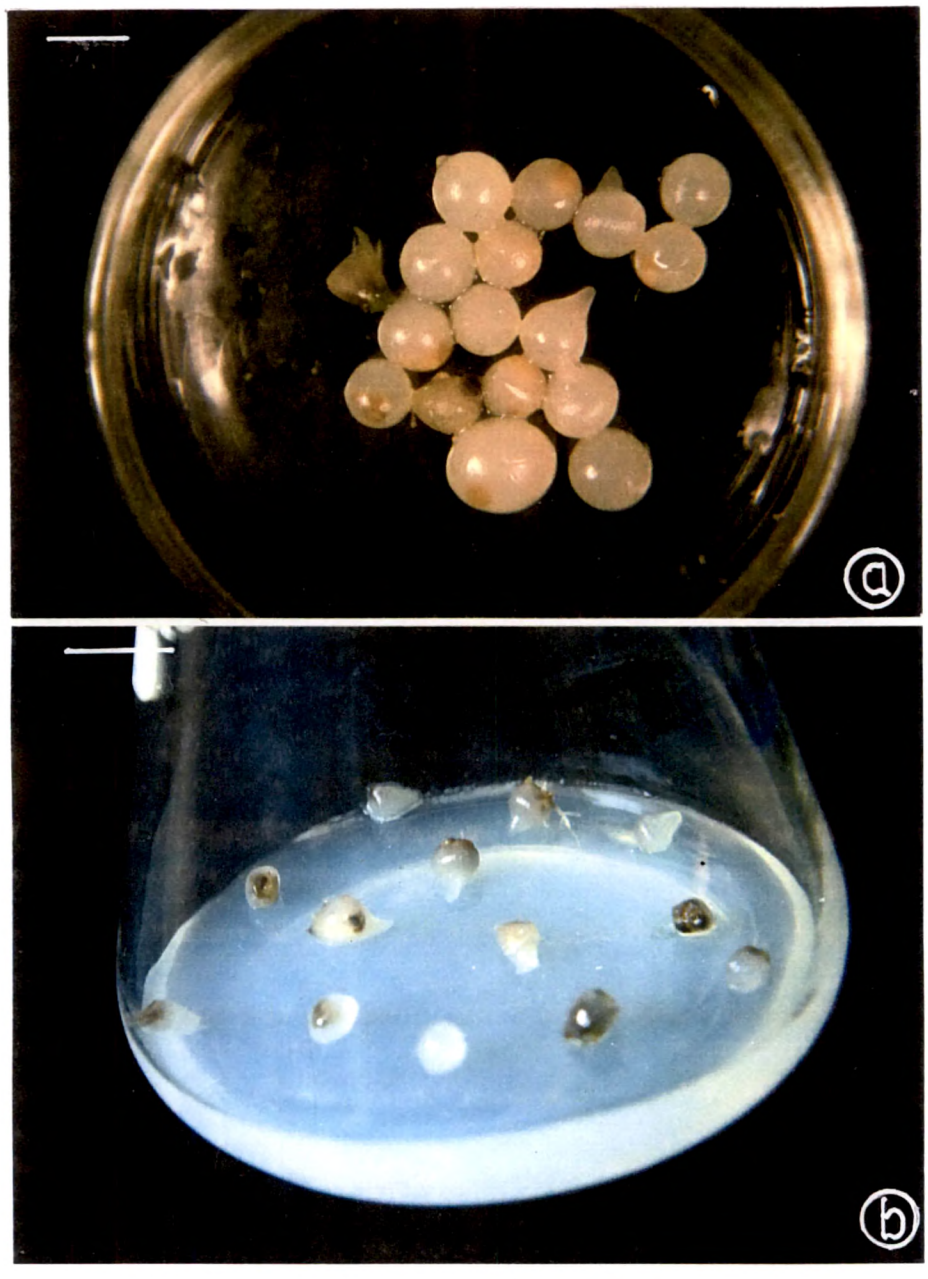


Plate 22

Plate 23 a) Plantlet emergence from encapsulated embryoids after 10 days of incubation

b) Adapted embryoid showing germination

c) Further growth of the plantlets from adapted embryoids

Bar = 1 cm

Plate 23

solid medium. The regenerants were transferred to pots for further growth (Plate 24, a and b). Occasionally albinos have also been observed in adapted cultures (Plate 21, a-c).

9) Storage and germination of encapsulated embryoids

The encapsulated embryoids when kept on germination medium (LS + 5% sucrose + 8mM Proline) germinated within 8-10 days (Plate 22 and 23). The embryogenic clumps showed slight bulging prior to emergence of the coleoptile or radicle. The encapsulated and non encapsulated embryoids did not showed any difference in their germination behaviour. The encapsulated control and salt tolerant embryoids at 25 ± 2°C showed 71.4 and 62.8% of germination (Table 21, Plate 23). Embryoids were stored at 6°C, 4°C and 2°C for a period of 10, 20 and 30 days. Encapsulated embryoids stored at 4°C for a period of ten days

Table 21: Effect of varying storage temperatures on percentage of germination in rice encapsulated embryoids. Germination medium used was 1/2 LS + 5% sucrose + 8mM proline.

Days after storage	Treatments	% of germination		
		Storage at 6°C	Storage at 4°C	Storage at 2°C
0	C	*71.2 ± 0.4	71.2 ± 0.4	71.2 ± 0.4
0	A	64.1 ± 0.7	64.1 ± 0.7	64.1 ± 0.7
10	C	22.4 ± 0.8	43.4 ± 0.1	13.4 ± 0.4
10	A	12.6 ± 0.7	21.3 ± 0.2	8.4 ± 0.8
20	C	17.8 ± 0.8	33.1 ± 0.4	8.7 ± 0.1
20	A	10.6 ± 1.4	18.4 ± 1.3	5.4 ± 0.1
30	C	13.4±0.7	23.1±0.4	12.3±0.2
30	A	10.2±1.23	16.3±0.3	9.2±0.2

C- Control, A-Adapted

* Mean ± S.E. values of three experiment with three replicates

Plate 24 a) Regenerants transferred to the pots

b) A two month old plant regenerated from adapted embryoids

Plate 24

showed higher percentage of germination. Prolongation of storage resulted in a reduction of germination percentage.

Biochemical studies

a) Free Proline content

The free proline content of embryogenic and non embryogenic calli under all treatment increased steadily and reached the highest level by day 40 (Table 22). Higher level of proline was observed in embryogenic calli

Table 22: Effect of LD_{50} concentration of NaCl on Proline content (mg g^{-1} fr. wt.) on Embryogenic callus (grown on LS +IAA)(0.1 mg/l) + BAP (0.5 mg/l) and Non-embryogenic callus (grown on LS medium supplemented (1.25 mg/l of 2,4-D) in rice

Type of the callus	Incubation period (in days)				
	0	10	20	30	40
NEC	*11.4±0.05^{a}	13.4±0.08^{a}	17.1±0.15^{a}	19.2±0.15^{a}	21.8±0.19^{a}
EC	18.4±0.13^{c}	21.4±0.14^{c}	24.8±0.18^{b}	27.1±0.11^{b}	29.2±0.15^{b}
NEA	16.1±0.08^{b}	16.2±0.8^{b}	17.8±0.1^{a}	28.4±0.12^{b}	38.4±0.61^{c}
EA	19.2±0.13^{c}	38.1±0.14^{d}	49.2±0.11^{c}	50.4±0.12^{c}	53.8±0.14^{d}

NEC - Nonembryogenic control
EC - Embryogenic control
NEA - Nonembryogenic Adapted
EA - Embryogenic Adapted

* Mean ± S.E. values of three separate experiments with three replicates. Values in the same column with the same superscript do not differ singificantly ($P \leq 0.05$) according to Duncan's Multiple Range Test.

compared to non-embryogenic ones. Under the influence of 0.25 M NaCl embryogenic and non-embryogenic calli registered 2.8 and 2.2 fold increase respectively compared to the initial level at zero day.

b) Total Protein content

The level of total protein in embryogenic and non-embryogenic calli increased gradually and reached the maximum on day 40 (Table 22). In contrast to the level of proline, protein contents of calli under the influence of NaCl showed a reduction.

Table 23 : Effect of LD_{50} concentration of NaCl on Protein content (mg g^{-1} fr. wt.) on embryogenic callus (grown on LS + IAA (0.1 mg/l) + BAP (0.5 mg/l) and non-embryogenic callus (grown on LS +1.25 mg/l of 2,4-D) in rice.

Type of the callus	Incubation period in days				
	0	10	20	30	40
NEC	*4.3±0.1[a]	4.8±0.13[a]	6.2±0.08[b]	9.4±0.13[d]	21.5±0.3[c]
EC	5.8±0.05[b]	6.4±0.2[b]	8.8±0.13[d]	10.4±0.1[d]	24.3±0.1[d]
NEA	4.3±0.11[a]	4.8±0.12[a]	5.4±0.1[b]	7.8±0.16[c]	15.3±0.08[b]
EA	4.4±0.3[a]	3.9±0.16[a]	4,.1±0.08[a]	5.2±0.3[b]	13.8±0.21[a]

NEC - Nonembryogenic control
EC - Embryogenic control
NEA - Non-embryogenic Adapted
EA - Embryogenic Adapted
* Mean ± S.E. values of three separate experiments with three replicates. Values in the same column with the same superscript do not differ singificantly ($P \leq 0.05$) according to Duncan's Multiple Range Test.

c) Total amylase activity

The total amylase activity exhibited a steady increase during the callus growth and registered the highest level at day 40 (Table 23). The enzyme activity decreased as a result of salinity. The embryogenic calli exhibited slightly reduced amylase activity compared to that of non-embryogenic calli.

Table 24: Effect of LD_{50} concentration of NaCl on the activity of amylase (unit mg-1 protein) in rice Embryogenic callus (grown on LS+IAA (0.1 mg/l) + BAP (0.5 mg/l) and Nonembryogenic callus (grown on LS+1.25 mg/l of 2,4-D) in rice

Type of the callus	Incubation period (in days)				
	0	10	20	30	40
NEC	*12.1 ±0.25^{c}	18.3 ± 0.2^{d}	25.4 ± 0.3^{d}	29.3 ± 0.4^{d}	36.1 ± 0.1^{d}
EC	10.2 ± 0.1^{b}	17.1 ± 0.11^{c}	21.3 ± 0.12^{c}	26.2 ± 0.2^{a}	31.8 ± 0.08^{c}
NEA	9.2 ± 0.13^{a}	12.4 ± 0.07^{d}	18.7 ± 0.1^{b}	22.4 ± 0.1^{b}	26.3 ± 0.5^{b}
EA	8.1 ± 0.13^{a}	10.3 ± 0.13^{a}	15.2 ± 0.07^{a}	18.3 ± 0.18^{a}	20.4 ± 0.16^{a}

NEC - Nonembryogenic control
EC - Embryogenic control
NEA - Non-embryogenic Adapted
EA - Embryogenic Adapted
* Mean ± S.E. values of three separate experiments with three replicates. Values in the same column with the same superscript do not differ singificantly ($P \leq 0.05$) according to Duncan's Multiple Range Test.

d) IAA-oxidase activity

The activity of IAA-oxidase in embryogenic and non-embryogenic calli rose steadily and reached a highest level on day 20th and decreased thereafter (Table 25). Embryogenic calli registered slightly higher activity compared to non-embryogenic calli.

Table 25 : Effect of LD_{50} concentration of NaCl on the activity of IAA oxidase (unit mg-1 protein) in rice Embryogenic callus (grown on LS+IAA (0.1 mg/l) + BAP (0.5 mg/l) and Nonembryogenic callus (grown on LS+1.25 mg/l of 2,4-D) in rice.

Type of the callus	Incubation period (in days)				
	0	10	20	30	40
NEC	*6.2± 0.1[a]	6.7± 0.17[a]	7.8± 0.08[a]	6.9± 0.00[a]	6.5± 0.8[a]
EC	9.2± 0.2[c]	9.7± 0.2[b]	9.8± 0.1[b]	8.2± 0.13[b]	7.4± 0.3[b]
NEA	8.3± 0.1[b]	8.3± 0.2[b]	16.1± 0.1[c]	9.4± 0.2[c]	7.1± 0.8[b]
EA	9.8± 0.8[c]	10.8± 0.2[c]	22.1± 0.12[d]	19.4± 0.08[d]	10.1± 0.19[c]

NEC - Nonembryogenic control
EC - Embryogenic control
NEA - Non-embryogenic Adapted
EA - Embryogenic Adapted
* Mean ± S.E. values of three separate experiments with three replicates. Values in the same column with the same superscript do not differ singificantly ($P \leq 0.05$) according to Duncan's Multiple Range Test.