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IN VITRO SELECTION FOR SALT TOLERANCE IN RICE

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ABSTRACT

Induced mutation in vitro was applied to increase levels of genetic variability in rice cell culture. Seeds of 12 varieties were cultured on various media for callus induction. It was found that the optimum medium was well responsed to callus production in Doc Do with MS + 2,4-D (2mg/l), Pokkali with MS+2,4-D (2mg/l); then Trang Thai Lan with MS+2,4-D (2mg/l), KDM105 with MS+2,4-D (2mg/l) to embryogenic callus formation ability. Parts of the calli were transferred to NaCl supplemented medium in an attempt to produce physiological variants from somaclones and evaluated some physiological aspects of cellular adaptation in response to salinity among genotypes selected in Soc nau, Doc Do, Trang Thai Lan, AS996, these increased the percentage of calli showing regeneation by 90.79%, 75.00%, 73.08%, and 68.75%, respectivity. This study was to create genetic variability in rice cell culture to obtain salt tolerance plants through in vitro selection. Futher attempts should be made to regenerate plants from these materials

Key words: *in vitro* selection, salt tolerance, somaclonal variation, tissue culture

INTRODUCTION

Soil salinity limits rice production in coastal regions of the Mekong delta. Tissue culture techniques have been widely used for breeding purpose, especially in selection for stress tolerance, tissue culture is a source of genetic variability that gives rise through genetic modifications during the process of *in vitro* culture, a phenomenon called somaclonal variation. The possible causes of somaclonal variation include chromosome aberrations, DNA amplification and the occurrence of transposable elements. This paper reports on the current status of tissue culture technology at CLRRI. Our primary objective is to demonstrate that useful germplasm can be obtained from tissue culture. A significant feature of this work is to determine what *in vitro* treatment, including concentration of the stressing agent and the period of time in culture, will produce the greatest probability of field-tolerant plant for a specific enviroment. This study was to create genetic variability in rice cell culture to obtain salt tolerance plants through *in vitro* selection.

MATERIALS & METHODS

Twelve rice cultivars were used in table 1 to conduct *in vitro* selection for salt stress

Table 1. Main features and origin of varieties

No	Designation	Original	Feature
1	IR42	IRRI	Mod. tol. to salinity, HYV
2	Pokkali (check)	India	Salt tolerance, landrace
3	AS996	IR64 / *O. rufipogon*	Tol. to acid sulfate soil, HYV
4	Nep Ao Gia	Vietnam	Mod. tol. to salinity, landrace
5	Trang Diep	Vietnam	Mod. tol. to salinity, landrace
6	Trang Thai Lan	Vietnam	Mod. tol. to salinity, landrace
7	Mong Chim Roi	Vietnam	Mod. tol. to salinity, landrace
8	IR29 (check)	IRRI	Supceplible to salinity, HYV
9	Khao Dawk Mali 105	Thailand	Good grain quality, landrace
10	Doc Phung	Vietnam	Mod. tol. to salinity, landrace
11	Doc Do	Vietnam	Mod. tol. to salinity, landrace
12	Soc Nau	Vietnam	Mod. tol. to salinity, landrace

HYV: high yielding variety, Mod. tol.: moderately tolerance

The medium for callus:

+ MS (Murashige & Skoog 1962) + 2mg/l 2-4D
+ MS (Murashige & Skoog 1962) + 1mg/l 2- 4D
+ N6 (Chu et al. 1976) + 2mg/l 2- 4D
+ N6 (Chu et al. 1976) + 1mg/l 2- 4D

The medium for regeneration:

+ MS (Murashige & Skoog 1962)
+ N6 (Chu et al. 1975)

1. MS+0,5mg/l NAA + 0,5mg/l BAP
2. MS+0,5mg/l NAA + 1mg/l BAP
3. N6+0,5mg/l NAA + 0,5mg/l BAP
4. N6+0,5mg/l NAA + 1mg/l BAP

NaCl medium

In vitro selection for salt stress tolerance was implemented at the concentrations of 0.5%, 1.0% and 1.5% NaCl.

The preparation of rice seeds for callus induction

Mature seeds were dehusked, surface sterilized with 70% ethylalcohol for 30 sec, and then for 30 min with 5% sodium hypochlorite (commercial bleach is mainly hypochlorite). Surface sterilized seeds were rinsed several times with sterile distilled water before inoculation on callus induction medium, consisting of MS basal organic and inorganic components (Murashige and Skoog 1962) supplemented with 3.0% sucrose, 2,4 D at 1.0- 2.0mg/l and kinetin at 0.0-0.5 mg/l (Lang 2002).

Culture conditions

The cultures were incubated under both dark and light conditions (16 hours day / 8 hours night). The temperature was maintained at 22^0C – 25^0C. The MS culture medium was supplemented with 3.0% sucrose, 0.8% agar, and various concentrations of growth regulators. The medium pH was adjusted to 5.8 prior to autoclaving at 15 psi for 20 minutes.

Media for callus induction and subculture

The basal medium for rice tissue culture used is similar to that developed by Murashige and Skoog (1962). Unlike many other crops, rice requires a unique growth regulator combination, sucrose concentration, and light regime for each cultivar. Culture vessels are either glass screw vails or jars. The vails contain 10ml of medium and are used for callus initiation. The jars containing 20ml of medium are use after embryogenic callus which was isolated and ready for plant regeneration.

For callus induction, the mature seeds are surface-sterilized and placed on the appropriate medium for that cultivar. At the end of 28 days – referred to as a "passage" – the original explant and all of its associated callus is transferred to a fresh medium. After the second passage, embryogenic (E) callus is isolated and transferred again to a fresh medium. Embryogenic callus is usually yellow or cream colored and dense in appearance. For longterm maintenance, embryocallus must be carefully selected and all nonembryogenic and dead callus removed at each transfer.

RESULTS

Time for callus initiation

Time for callus initiation was noticed from 5 to 10 days after placing seeds on agar medium among 12 rice cultivars (table 2).

Table 2. The time for callus initiation (days) for 12 rice cultivars

Cultivar	The time for callus initiation (days)	Cultivar	The time for callus initiation (days)
1. IR42	10	7. Mong Chim Roi	5
2. Pokkali	5	8. IR29	10
3. AS996	10	9. Khao Dawk Mali 105	7
4. Nep Ao Gia	5	10. Doc Phung	10
5. Trang Diep	5	11. Doc Do	7
6. Trang Thai Lan	7	12. Soc Nau	10

Ability of callus formation

The ability to form callus of cultivars on various media offered different results

1 Very good I MS+2,4-D(2mg/l)
2 Good II MS+2,4-D(1mg/l)
3 Medium III N6+2,4-D(2mg/l)
4 Average IV N6+2,4-D(1mg/l)
5 Dead

Table 3. The effect of media and cultivars on embryogenic callus formation ability

Cultivars	Medium for culture				Effect of cultivars
	MS+2,4-D (2mg/l)	MS+2,4-D (1mg/l)	N6+2,4-D (2mg/l)	N6+2,4-D (1mg/l)	
1. IR42	3.000 fg	4.000 i	3.667 hi	4.000 i	3.667 e
2. Pokkali	1.000 a	2.000 cd	1.333 ab	3.333 hg	1.917 ab
3. AS996	3.333 gh	3.333 gh	3.333 gh	3.667 hi	3.417 de
4. Nep Ao Gia	1.667 bc	2.333 de	2.333 de	3.000 fg	2.333 abc
5. Trang Diep	3.000 fg	3.333 gh	2.667 ef	3.333 gh	3.083 cde
6. Trang Thai Lan	1.333 ab	2.667 ef	3.667 hi	3.333 gh	2.750 bcde
7. Mong Chim Roi	1.667 bc	2.333 de	2.333 de	3.333 gh	2.417 abc
8. IR29	3.000 fg	3.000 fg	2.333 de	3.667 hi	3.000 cde
9. KDM 105	1.333 ab	3.333 gh	1.667 bc	3.667 hi	2.500 abcd
10. Doc Phung	2.000 cd	3.333 gh	3.000 fg	3.333 gh	2.917 cde
11. Doc Do	1.000 a	1.333 ab	2.333 de	2.333 de	1.750 a
12. Soc Nau	2.000 cd	2.333 de	2.000 cd	2.333 de	2.167 abc
Effect of media	2.028 a	2.778 a	2.556 a	3.278 a	
					CV = 19,68%

The means have the same letter following is not significant difference at 0.01 probability level

Table 3 shows that in the effect of media on embryo callus formation, the medium MS+2,4-D (2mg/l) was not significantly different from other media. For the effect of cultivars, Doc Do was considered as the best variety then Pokkali, Soc Nau, Nep Ao Gia, Mong Chim Roi, KDM105 were also noticed as more suitable genotypes on embryogenic callus formation ability as compared to others.

In the effect of media and cultivars interaction, the treatments Doc Do x MS+2,4-D(2mg/l), Pokkali x MS+2,4-D(2mg/l) obtained very good result; Trang Thai Lan x MS+2,4-D(2mg/l), KDM105 x MS+2,4-D (2mg/l) obtained good result on embryogenic callus formation ability

The average percentages of embryogenic callus formation.

The effect of various concentrations of 2,4-dichlorophenoxy acetic acid (2,4-D) of media on callus induction was presented in table 4. Callus initiation was observed after 3 days of culture in all cultivars, irrespective of the light or dark period.

Table 4. The effect of media on percentage of embryogenic callus formation

Medium	Percentage of embryogenic callus formation (%)
MS+2,4-D(2mg/l)	55.56 a
MS+2,4-D(1mg/l)	47.78 ab
N6+2,4-D(2mg/l)	37.78 ab
N6+2,4-D(1mg/l)	29.72 b
CV	16.89%

The result showed that medium MS+2,4-D(2mg/l) was more suitable to embryogenic callus formation than the others (table 4).

The effect of cultivars on embryogenic callus formation percentage is presented in table 5. Doc Do, Soc Nau, Nep Ao Gia, AS996 were genotypes exhibited more suitable to develop in cultural medium than others.

Table 5. The effect of cultivars on percentage of embryogenic callus formation**.**

Designation	Percentage of embryogenic callus formation (%)
1. IR42	29.17 fg
2. Pokkali	45.00 d
3. AS996	50.83 c
4. Nep Ao Gia	63.33 b
5. Trang Diep	39.17 e
6. Trang Thai Lan	32.50 f
7. Mong Chim Roi	24.17 gh
8. IR29	27.50 fg
9. Khao Dawk Mali 105	20.83 h
10. DocPhung	43.33 de
11. Doc Do	73.33 a
12. Soc Nau	63.33 b
CV	16.89

The means have the same letter following is not significant difference at 0.01 probability level

The effect of media and cultivars interaction on embryogenic callus formation percentage is presented in table 6. Treatments Doc Do x MS+2,4-D (1mg/l); Nep Ao Gia x MS+2,4-D (2mg/l) obtained percentage of embryogenic callus formation 86.67% and 80% significantly different at 0.01 probability level, respectively

Table 6. The effect of media and cultivars interaction on percentage of embryogenic callus formation

Designation	Culture medium			
	MS+2,4-D (2mg/l)	MS+2,4-D (1mg/l)	N6+2,4-D (2mg/l)	N6+2,4-D (1mg/l)
1. IR42	36.67 jk	26.67 mn	26.67 mn	26.67 mn
2. Pokkali	66.67 cd	53.33 g	36.67 jk	23.33 no
3. AS996	63.33 de	63.33 de	40.00 ij	36.67 jk
4. Nep Ao Gia	80.00 b	70.00 c	56.66 fg	46.67 h
5. Trang Diep	56.67 fg	43.33 hi	36.67 jk	20.00 o
6. Trang Thai Lan	46.67 h	30.00 lm	33.33 kl	20.00 o
7. Mong Chim Roi	43.33 hi	23.33 no	20.00 o	10.00 p
8. IR29	46.67 hi	33.33 kl	20.00 o	13.33 p
9. KDM105	26.67 mn	23.33 no	20.00 o	13.33 p
10. Doc Phung	56.67 fg	53.33 g	33.33 p	30.00 lm
11. Doc Do	80.00 b	86.67 a	66.67 p	60.00 ef
12. Soc Nau	66.67 cd	66.67 cd	63.33 p	56.67 fg
CV (%)	16.89			

The means have the same letter following is not significant difference at 0.01 probability level

In conclusion, Doc Do, Nep Ao Gia, and the other genotypes as Pokkali, Soc Nau were also suitable to embryogenic callus formation. The optimum medium for embryogenic callus formation was MS+2,4-D(2mg/l).

Regeneration

Non-embryogenic (NE) callus was not regenerable when transferred to the regeneration MS medium supplemented with 1.0mg/l benzylaminopurine (BAP) and 0.5mg/l of naphthalene acetic acid (NAA). On the other hand, when embryogenic (E) callus was transferred to the regeneration medium, root initiation and callus differentiation was observed within one week of incubation. Visible shoot formation was noted four weeks later on the cultivar on Trang Thai Lan (82 shoots per E callus from 0.5 to 1.0 cm, the average percentages of E callus form shoot were 18%) and Trang Diep (3 shoots per E callus, the average percentages of E callus form shoot were 18%). The other cultivars are continuous observable.

***In vitro* selection for salt stress tolerance**

For salt treatment studies, attempts to produce embryogenic calli were conducted in saline medium as 0.5%, 1.0% and 1.5% NaCl which were added to the callusing medium specific for each genotype, as per results obtained in the initial experiment. Induced calli were excised two weeks after inoculation of explants, transferred to fresh medium for proliferation for a peroid of 21 days, and then to the regeneration medium.

1.5%NaCl added to the regeneration medium increased the percentage of calli showing regeneation by 90.79%, 75.00% in Soc Nau and Doc Do (figure 1). In Trang Thai Lan, AS996 and Pokkali, 73.08%, 68.75% and 51.28% reduction in the number of regenerating calli were recognized. Salts tress sensitive genotype IR 29 was not clearly responsed (table 7).

Table 7. Plant regeneration of rice calli under NaCl stress

Varieties	% callus regeneration		
	0.5% NaCl	1% NaCl	1.5%NaCl
Pokkali	100.00	100.00	51.28
AS996	100.00	55.56	68.75
Nep Ao Gia	100.00	100.00	60.00
Trang Diep	92.86	74.21	55.56
Trang Thai Lan	100.00	100.00	73.08
Mong Chim Roi	100.00	100.00	63.64
KDM105	75.00	92.86	44.12
Doc Phung	100.00	100.00	64.00
Doc Do	100.00	100.00	75.00
Soc Nau	100.00	100.00	90.79
IR 29 (check)	100.00	78.57	47.62

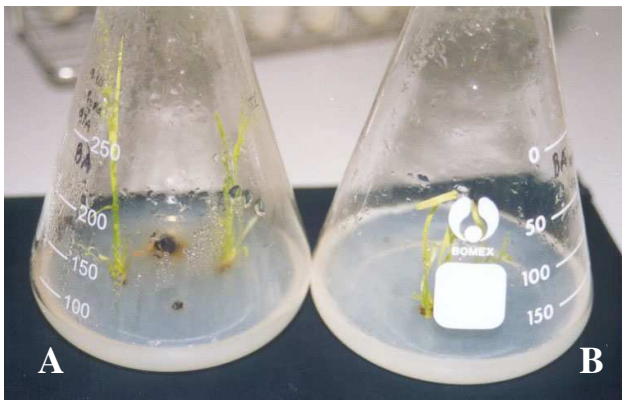

Figure 1: Plant regeneration on non salinized (A) and salinized regeneration medium (B) in Pokkali genotype

CONCLUSION & SUGGESTION

MS+2,4-D(2mg/l) is considered as a medium suitable for target cultivars to culture.

The genotypes as Doc Do, Nep Ao Gia, and the others as Pokkali, Soc Nau, Mong Chim Roi, KDM105 are also suitable for embryogenic callus formation.

The regeneration MS medium sup-plemented with 0.5mg/l benzylamin-opurine (BAP) and 1.0mg/l of naphthalene acetic acid (NAA) effectively affected Trang Thai Lan (82 shoots per E callus from 0.5 to 1.0 cm, obtained 18% E callus form shoot) and Trang Diep (3 shoots per E callus, obtained 18% E callus form shoot). The other cultivars have been continously obverved.

In medium added NaCl 0.5%, E callus of all cultivars developed normally. Both treated and non–treated calli that survived in 1.0 and 1.5% NaCl were of considerable benefit to further studies. Futher attempts should be made to regeneate plants from these materials. Suggestion to tests for salinity response in the salt tolerant Doc Do that R_0, R_1 progenies.

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SUMMARY IN VIETNAMESE

Khai thác biến dị sô ma các dòng lúa chống chịu mặn từ nuôi cấy *in vitro*

Khai thác đột biến trong môi trường nuôi cấy mô là một kỹ thuật được khuyến cáo để tìm ra những biến dị di truyền có lợi, đặc biệt là chọn lọc giống lúa chống chịu mặn. Mười hai giống lúa bao gồm giống chống chịu và giống nhiễm mặn đã được nghiên cứu. Môi trường tối hảo cho sản sinh mô sẹo là MS + 2,4-D(2mg/l) đối với giống Đốc Đỏ, MS+2,4-D(2mg/l) đối với giống Pokkali; cho khả năng tạo mô sẹo ở phôi là MS+2,4-D(2mg/l) đối với giống Trắng Thái Lan, MS+2,4-D(2mg/l) đối với giống KDM105. Chuyển mô sẹo sang môi trường có chứa NaCl như một yếu tố stress do mặn. Các nghiệm thức của giống Sóc nâu, Đốc Đỏ, Trắng Thái Lan, AS996 có tỉ lệ cây tái sinh cao nhất 90.79%, 75.00%, 73.08%, và 68.75%, theo thứ tự. Nghiên cứu này mở ra triển vọng khai thác nguồn biến dị di truyền từ tế bào sô ma và chọn lọc dòng chống chịu *in vitro* một cách có hiệu qủa. Vật liệu này sẽ được tiếp tục nghiên cứu đánh giá trên đồng ruộng.
