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ENHANCED SOMATIC EMBRYOGENESIS UNDER SALT STRESS CONDITION IN *JUSTICEA GENDARUSSA*

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SUMMARY

Somatic embryogenesis is a desirable morphogenetic pathway that allows regeneration of plantlets in a single step, thereby avoiding the complicated rooting step to recover plants from regenerated shoots. Leaf explants of *Justicea gendarussa* were inoculated on Murashige and Skoog's medium (Murashige and Skoog 1962) containing 2mg/l 2, 4-D and different concentrations of one of the three salts-NaCl, KCl, Na_2SO_4. Explants were subjected to salt stress in two ways- gradual treatment and shock treatment. The callus produced in both cases were subcultured using a diverse range of media, with and without salt and it was found that the morphogenetic path that the surviving cells adopted was somatic embryogenesis. Somatic embryos were formed in large numbers in the salt tolerant calli and showed bipolarity on germination.

Key words: medicinal value, salt stress, *in vitro*, tolerance, somatic embryos

INTRODUCTION

Salinity is a major factor limiting crop productivity. Broadly it can be dealt with by using technological advances in water and soil management, irrigation methodology and/or through biological approaches such as breeding of resistant varieties or cultivation of naturally salt tolerant crops.

Conventional plant breeding methods have met with limited success in developing varieties tolerant to salt stress. Inadequate genetic sources for salinity tolerance, lack of efficient screening procedures etc. have hindered progress on this front. Tissue culture approach involving *in vitro* selection of salt tolerant cell lines has been reported in a large number of plants, viz. rice, alfalfa, *Pennisetum purpureum* (Ben-Hayyim *et al.* 1989).

Skoog and Miller (1957) promulgated that the ratio of auxin and cytokinin in the medium determined the type and extent of organogenesis. Propagation of a selected genotype is possible by encouraging the pre-existing meristem towards growth and proliferation by shoot morphogenesis, either directly from the explant or from the unorganized callus or by somatic embryogenesis, which may again be direct or indirect.

This report describes the regenerative path adopted during *in vitro* selection to screen directly for salt tolerant cell lines of *Justicea gendarussa*, commonly known as Jagat madan and economically important in ayurveda for treatment of lunacy, debility, snakebite, amenorrhoea and stomach troubles.

MATERIALS AND METHODS

Initiation and maintenance of callus line

Leaf explants of *Justicea gendarussa* were surface sterilized with 70 % alcohol and 0.1 % $HgCl_2$ (1 min.) and

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finally rinsed thoroughly with autoclaved distilled water. They were inoculated in MS (Murashige and Skoog 1962) medium containing 2mg/l 2,4-D as it was found to produce maximum callus in all cultures. The pH was adjusted to 5.7±0.1 prior to autoclaving at 121 °C for 15 min. The cultures were maintained at 25°C under 16 h daily illumination of 1500 lux from cool, white fluorescent lamps. Callus produced was green and friable and used as control while screening for salt tolerance.

Screening for salt tolerance

Equal sized explants (1cm^2) from leaves of same age were inoculated on MS medium having 2mg/l 2, 4-D and supplemented with different concentrations of the salt. Three salts- NaCl, KCl and Na_2SO_4 were used individually in concentrations of 0.1, 0.5, 1, 2, 3, 5, 7, 10 and 20 g/l for screening for salinity resistance. The low concentrations of 0.1, 0.5, 1 and 2 g/l comprised the gradual treatment while the higher concentrations were taken as shock treatment.

Maintenance of salt tolerant callus

The surviving calli out of gradual and shock treatments were either subcultured on the same medium, where very slow growth was observed or were transferred to basal MS medium without any additional salt.

Test for sustenance of tolerance

The callus lines tolerant to additional salt in the medium were tested for retention of the tolerance after having been maintained on MS medium having 2 mg/l 2, 4-D for 4-6 weeks. They were subcultured on salt containing medium and the growth rate was assessed. Only 45 % of the cultures survived and these were used for inducing morphogenesis.

Regeneration

The callus obtained by gradual and shock treatment on 2, 4-D containing medium were subcultured on medium with altered growth regulators, to induce a regenerative pathway. Combinations of BAP and NAA were able to induce somatic embryogenesis. Though the callus did not significantly increase in amount, it was directed towards the development of somatic embryos.

RESULTS AND DISCUSSION

The response of leaf and node explants of *Justicea* was observed in preliminary experiments in a wide range of combinations of growth regulators and the morphogenetic requirement of both were found to vary. The node explants regenerated shoots from the pre-existing meristem in MS medium containing NAA and kinetin, while the leaf explants showed indirect organogenesis, by development of shoots from a proliferating callus mass (Fig. 1, 2 & 3). In both rooting had to be induced by transfer to another medium containing 2 mg/l IBA. Best callus was obtained in 2, 4-D (2 mg/l) containing MS medium for leaf explants and this medium was used for the addition of salt to select tolerant cell lines.

Justicea leaf explants invariably produced callus in MS medium supplemented with 2 mg/l 2, 4-D, 7 g/l agar, 30 g/l sucrose and varying concentrations of any of the three salts - NaCl, KCl and Na_2SO_4 (Table 1). The control cultures, without salt, produced sufficient, soft and greenish white callus in 30 days with initiation starting in 2-3 days. At 0.1, 0.5, 1, and 2 g/l NaCl curling of leaf explants initiated in 3-4 days. They formed callus after 30 days. The initial response was slow but gradually growth was induced in more cultures. At 3 and 5 g/l NaCl, callusing initiated in 2-3 days, while in 10 g/l NaCl brownish callus, poor in amount initiated in 6-7 days in all the cultures, but with very poor survival.

With KCl, the response was a little delayed. KCl at 0.1g/l initiated the response in 3-5 days in the form of dense and white callus. At 0.5 and 1 g/l KCl the response was initiated as curling of leaf explant in 4-6 days, which produced poor white callus. KCl at 2 and 3g/l showed initiation of callusing in 4-6 days and produced brown callus. With the increase in concentrations to 5, 7, 10 and 20g/l, there was 7-8 days increase in days to initiation and colour of the callus changed from cream to pinkish brown. Though initiation took place in all the cultures, the survival frequency was very low, especially at 10 and 20 g/l KCl in the medium.

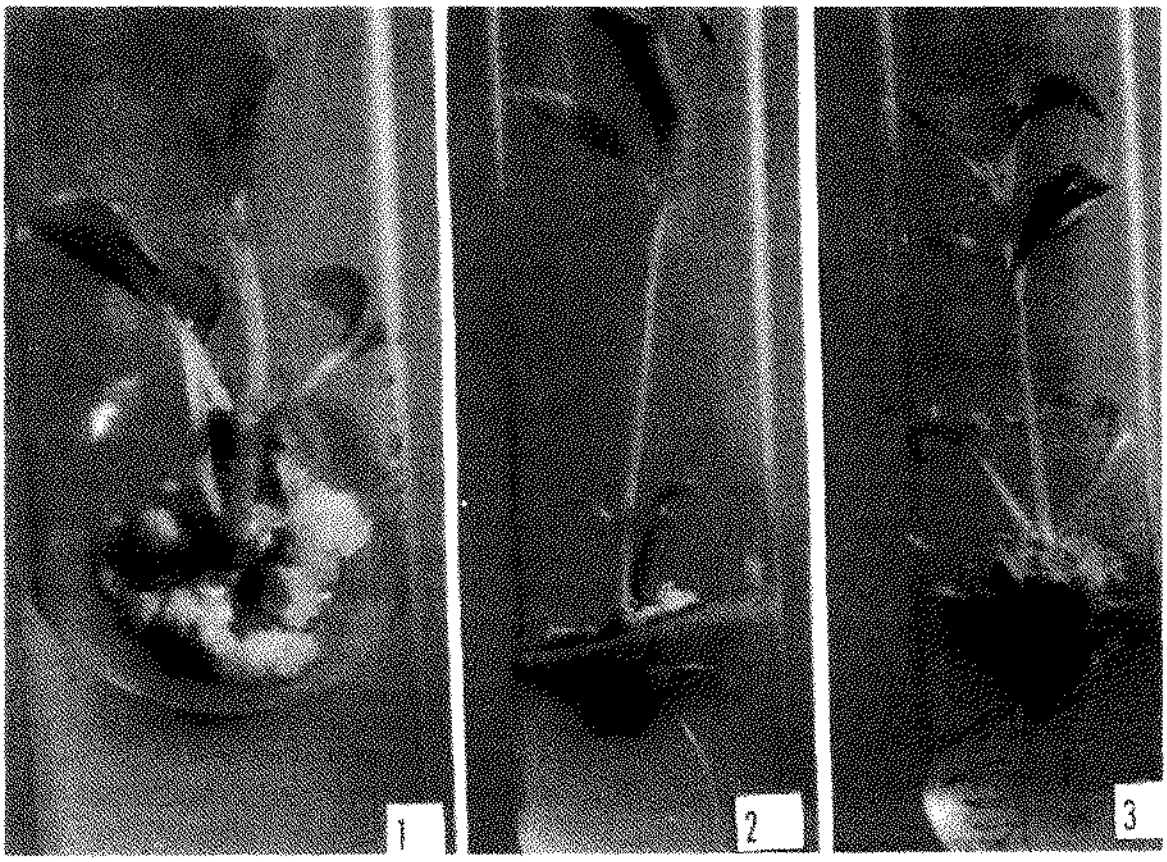

Fig. 1. Multiple shoots from node explant in MS medium containing NAA (1mg/l) and kinetin (2mg/l)
Fig. 2. Plantlet from node explant in above medium
Fig. 3. Plantlet from leaf explant in above medium

Table 1. Callusing response of leaf explants of *Justicea gendarussa* in varying concentrations of salts in MS medium supplemented with 2mg/l 2,4-D.

Salt g/l	NaCl			KCl			Na_2SO_4		
	Days to initiate	% response	% survival	Days to initiate	%response	% survival	Days to initiate	%response	% survival
0	2-3	100	100	3-4	100	100	3-4	100	100
0.1	3-4	75	40	3-5	80	80	3-4	80	75
0.5	3-4	75	40	4-6	80	80	4-5	65	60
1.0	3-4	70	40	5-6	80	75	4-5	75	60
2.0	3-4	80	50	5-6	90	75	5-7	80	75
3.0	2-3	100	87	4-5	100	62	4-5	100	70
5.0	2-3	100	87	4-5	100	50	6-7	90	65
7.0	3-4	100	62	5-7	100	40	7-8	90	50
10.0	6-7	100	37	7-8	100	32	8-9	100	37
20.0	6-7	100	37	7-8	100	12	8-9	100	12

Na_2SO_4 supplemented MS medium further delayed the response. Na_2SO_4 at 0.1 g/l initiated curling in 3-4 days and after 30 days produced good amount of cream coloured callus. Na_2SO_4 at 0.5, 1 and 2 g/l initiated callusing in 4-7 days to produce substantial callus of white colour. Na_2SO_4 at 3 g/l initiated response in 4-5 days to produce sufficient callus of white colour after 30 days. The days to initiation were increased to 8-9 days and the amount became poor. The colour changed from cream to brown.

In general, the three salts induced almost similar response with high survival at low concentration used for gradual treatment and poor survival at high concentration used for shock treatment. Low concentrations of salt in medium were able to induce response almost similar to control, sometimes even better. The callus raised on low salt medium was subjected to higher salt ranges in a step wise manner, climbing each concentration at a time. The survival decreased at each increasing concentration but was more than that obtained by direct inoculation of callus at higher level. The percentage survival at high salt concentration was extremely low with the developing callus being hard and brownish with a very slow growth rate. When transferred to basal medium the callus showed slight improvement in growth.

The callus obtained by gradual and shock treatment on 2, 4-D containing medium were subcultured on changed medium with altered growth regulators, to induce a regenerative pathway. Combinations of BAP and NAA were able to induce somatic embryogenesis. Though the callus did not significantly increase in amount, it was directed towards the development of somatic embryos. This initiated in the form of dense and compact cell growth in the surface region followed by differentiation of shiny globular bodies, and their microscopic examination confirmed their bipolar nature. These somatic embryos were capable of independent germination when transferred to fresh medium. Salt tolerance can be considered as the nature of embryogenic cells, which tolerate higher levels of salt stress than the other cell type (Table 2, Figs.4-9).

At the cell level, the response can be affected by different factors such as culture medium and the explant source from which callus was derived (Garcia-Reina *et al.* 1988). A possible factor could be the large variation generated during culture, known as somaclonal variation, which could contribute to modifications in the degree of salt tolerance. It has been shown that plant cells *in vitro* have a high adaptation capacity, which increases with the subculture number.

The acquisition of salt tolerance by any plant is difficult to achieve because of complex interaction between plant system and changing environmental conditions. There have been successful attempts in development of callus lines tolerant to NaCl stress (Pandey and Ganapathy 1985). Gosal and Bajaj (1984) undertook studies to compare NaCl tolerance at different levels of plant organisation. *In vitro* growth response indicated that callus growth was inhibited at 1% and 3% NaCl but it was not reduced to zero.

Induction of somatic embryogenesis requires a change in the fate of a vegetative (somatic) cell. In most cases, an inductive treatment is required to initiate cell division and establish a new polarity in the somatic cell. In alfalfa, the inductive treatment is most commonly 2, 4-D but other auxins such as 2,4,5-T are also effective. The auxin response is quite complex (McKersie and Brown 1996), while some auxins such as IAA and IBA are ineffective, and still others will stimulate the formation of embryos and callus but not somatic embryos. Inorganic components in the medium such as potassium, and organic components such as proline can modulate the embryogenesis or callus response, but they can not replace auxin (Shetty and McKersie 1993).

According to Feher *et al.* (2002) plant ontogenesis has a remarkable plasticity with continuous post embryogenic organogenesis during the entire life cycle. This is due the presence of specific undifferentiated organ forming lines called meristems, the activity of which is maintained, initiated or stopped by endogenous as well as environmental signals. It is generally accepted that the reactivation of cell division in somatic plant cells is required for dedifferentiation (Nagata *et al.* 1994) and the establishment of embryogenic competence (Dudits *et al.* 1991, 1995, Yeung 1995). Auxin is considered to be the main plant hormone required for the activation of cell division in differentiated plant cells both *in vivo* and *in vitro*. It is important in relation to cell division and differentiation as well as the induction of somatic

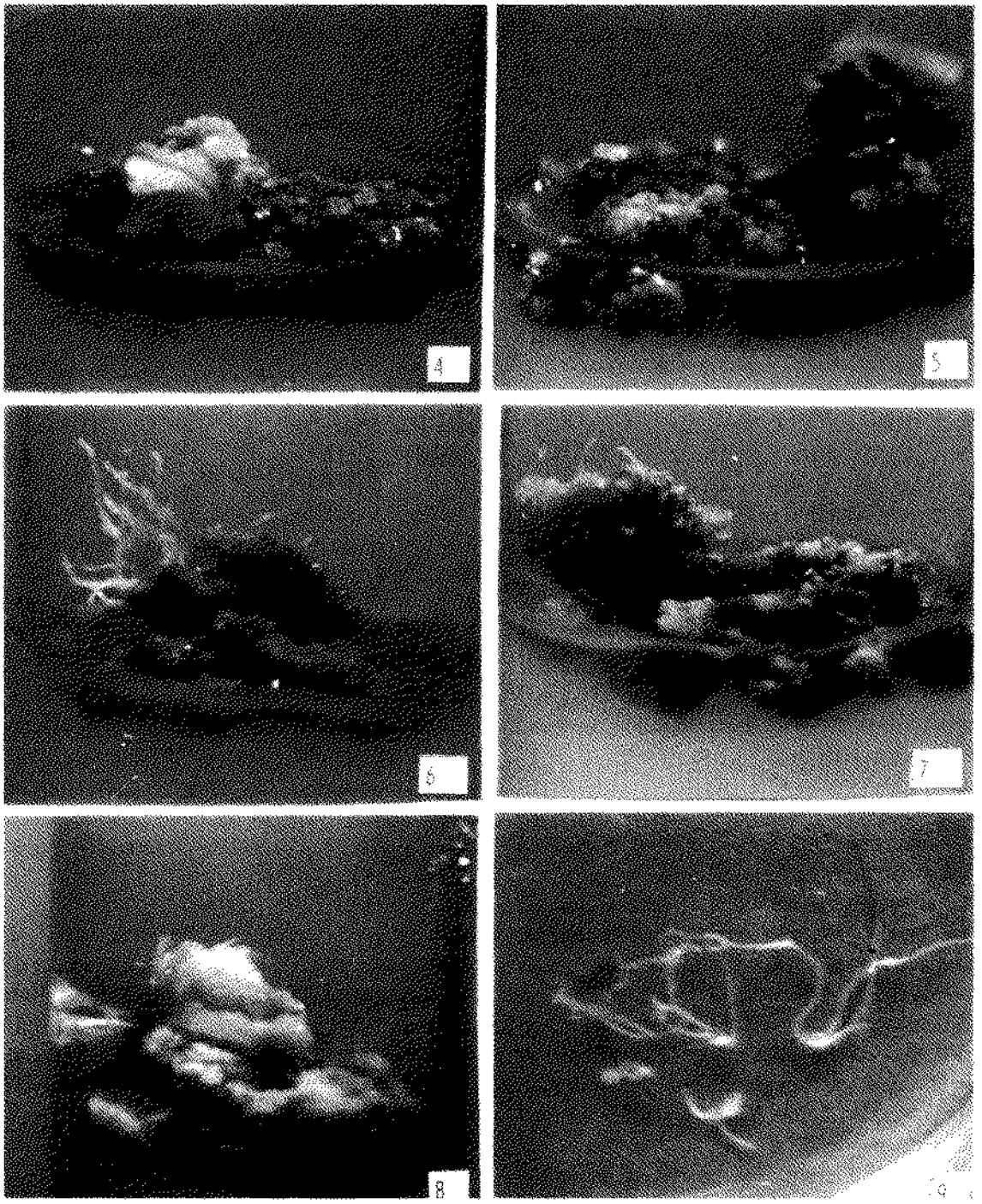

Fig. 4. Embryogenic callus (tolerant line on 0.5g/l maintained on basal medium and transferred to 2 g/l NaCl)
Fig. 5. Embryogenic callus (tolerant line on 2g/l maintained on basal medium and transferred to 3 g/l NaCl)
Fig. 6. Embryogenic callus (tolerant line on 3 g/l maintained on basal medium and transferred to 3 g/l NaCl)
Fig. 7. Embryogenic callus (tolerant line on 2 g/l maintained on basal medium and transferred to 3 g/l KCl)
Fig. 8. Embryogenic callus (tolerant line on 3g/l maintained on basal medium and transferred to 3g/l KCl)
Fig. 9. Bipolar somatic embryos

Table 2. Embryogenic potential of salt tolerant callus lines maintained on basal medium on transfer to salt containing medium (* Average number of somatic embryos per culture)

MS medium		NaCl			KCl			Na_2SO_4		
Growth regulator	Salt conc. g/l	% callusing	%with SE	No. of SE*	% callusing	% with SE	No. of SE*	% callusing	%with SE	No. of SE*
2,4-D 2mg/l	0	76	30	8	80	-	-	82	-	-
	0.1	76	32	17	75	-	-	78	-	-
	0.5	70	40	18	75	-	-	70	-	-
	1.0	71	42	28	65	31	12	62	20	17
	2.0	48	45	34	54	38	15	62	20	20
	3.0	41	43	17	50	45	20	48	24	25
	5.0	35	58	17	32	50	21	32	40	23
	7.0	30	20	6	30	54	16	30	34	9
	10.0	16	08	6	10	21	4	14	-	-
	20.0	09	-	-	6	10	4	14	-	-
BAP(1mg/l) NAA(1mg/l)	0	78	12	4	78	-	-	81	-	-
	0.1	70	23	7	75	-	-	72	-	-
	0.5	70	35	15	62	10	4	65	-	-
	1.0	71	42	15	60	24	7	61	46	5
	2.0	75	48	29	62	27	7	65	45	8
	3.0	63	43	10	55	34	9	55	53	8
	5.0	45	22	9	57	45	18	42	55	18
	7.0	40	8	3	32	22	18	45	20	10
	10.0	15	-	-	12	20	15	20	13	3
	20.0	15	-	-	08	-	-	15	-	-

embryogenesis. 2, 4-D has been shown to influence the endogenous auxin (indole acetic acid) metabolism in carrot cells, which was hypothesized to affect somatic embryogenic transition (Michalczuk *et al.* 1992). Pasternak *et al.* (2000) elucidated the role of auxin, pH and stress on the activation of embryogenic cell division in leaf protoplast derived cells of alfalfa (*Medicago sativa* L.) and reported similar effects on the endogenous IAA level when the cells were cultured under stress conditions (iron stress). The formation of embryogenic cell type is enhanced by different stress treatments like iron, copper, paraquat, menadion etc. (Feher *et al.* 2002) when non embryogenic cells are cultured in low 2, 4 –D containing medium.

Our results reveal that callus lines selected on the basis of their ability to grow under salt stress, when transferred to suitable growth medium for regeneration of plantlets, show the enhanced development of somatic embryos. Under normal conditions, adventitious buds were formed for regeneration while under salt stress the surviving callus cells get programmed due to endogenous changes *e.g.* increased proline, to develop somatic embryos.

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