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IDENTIFICATION OF MARKERS FOR NaCl TOLERANCE IN CELL LINES OF *JATROPHA CURCUS*- A BIO-DIESEL PLANT

MANISHA DEWAN, ASHOK K. DHAWAN* AND HANS R. DHINGRA**

Department of Botany and Plant Physiology, CCS Haryana Agricultural University, Hisar-125 004

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SUMMARY

Sub-culturing of leaf callus on Murashige and Skoog's (MS) medium with 2.5 x 10^{-6}M á-naphthalene acetic acid (NAA) and 5 x10^{-6}M each of 6-benzylaminopurine (BAP) and kinetin (KIN) for six generations supplemented with NaCl (105mM) or without NaCl yielded six different callus lines viz. control, unadapted salt stressed and salt adapted. Adapted calluses were first shifted to NaCl free medium (adapted →C) and later to salt supplemented medium (adapted →C→S). SDS-PAGE analysis revealed that polypeptides of MW 1000, 79.4 and 67.6 kDa, synthesized in response to NaCl are critical for survival of cells under salt stress. RAPD analysis of salt exposed variants showed that primers 100C04, 90B11 and BG56S110C15 can possibly be employed to detect amplification products related to salt tolerance in *Jatropha.*

***Key words: In vitro, Jatropha curcus,* protein markers, RAPD, salt tolerance**

INTRODUCTION

Salt stress has become an ever increasing constraint to food production since irrigation continues to cause gradual build up of salinity in arable lands (Rabie and Almadini 2005). Zhu (2001) reported that 20% of the world's cultivated and nearly 50% of irrigated land is affected by salinity. This loss of farmable land is in direct conflict with the needs of world population projected to increase by 1.5 billion in the next 20 years. Salinity also limits the caloric and the nutritional potential of agricultural production (Sureena *et al.* 1999, 2001). Reclamation of these soils offers one of the possible solutions but this is quite expensive and seldom effective. Such a predicament advocates for evolving salinity tolerant varieties for improved utilization of saline soils and water. Salinity tolerance is a quantitative trait (Foolad and Jones 1993) and has generally been resistant to improvement by conventional plant breeding. Tissue culture technique offers an alternative route for improvement of salinity tolerance through selection of somaclonal variants (Tal 1994, Rus *et al.* 2000). Selection pressure in this technique is applied to a large population of somatic cells to unravel the desired phenotype (Hasegawa *et al.* 1994).

Studies have evinced that salt tolerance is acquired when cultures are exposed to a stress agent for several passages (Hsissou and Bouharmont 1994, El-Hannawy 1996). For an *in vitro* selected trait to prove agriculturally useful, it should be stable in the absence of stressing agent and the cell lines carrying that trait should be easy to propagate. Moreover, *in vitro* selection for salt stress can be of great use in elucidating the mechanism of salt tolerance at cellular level (Piqueras and Hellin 1992).

Jatropha curcus L. (Physic nut), a plant species belonging to family Euphorbiaceae, is a native of Mexico

**Corresponding author, hrdhingra@hau.nic.in, hrdhingra2001@yahoo.co.in; *Regional Research Station, CCS HAU, Uchani, Karnal-132 001

and tropical South America which has naturalized now throughout tropical to subtropical Asia and Africa. It grows luxuriantly in marginal, stony and sandy tracks with low nutrients and thus well-adapted to arid and semiarid conditions. *Jatropha* is considered as a promising biofuel crop of wastelands, since its seeds are enriched with 28-35% viscous oil. Dry climate is known to greatly improve the oil yield of the seeds. In Asia and Africa physic nut is used in traditional medicines as a laxative, emetic, cough treatment and for healing wounds (Nath and Dutta 1992). In view of multifarious uses of *Jatropha curcus*, more particularly as a biofuel crop and being endowed with rare virtue of drought tolerance, the present study was undertaken to induce salt tolerance *in vitro* and to decipher biochemical, molecular and physiological basis of salt tolerance.

MATERIALS AND METHODS

Callus was raised from leaf lamina explants (from two years old healthy plants) on MS medium supplemented with 2.5 x 10^{-6} M NAA and 5 x 10^{-6} M each of BAP and KIN. Callus pieces, weighing approximately 50 mg, were subcultured on the above medium with or without salt. Entire experiment was divided into five different sets. In the first set, calli were repeatedly cultured on NaCl free medium at an interval of 28 d for six generations. The callus line raised on this medium served as control. In the second set, calli from some of these flasks after 5 subcultures were shifted to the medium supplemented with 105 mM NaCl for sixth sub-culture (unadapted salt stressed callus line). In the third set, calli were sub-cultured on salt supplemented medium repeatedly for six generations (adapted callus line). A sizeable number of adapted calli after five successive subcultures on salt supplemented medium were shifted to the NaCl free medium, to see whether acquired salt acclimation is transient or a permanent character (adapted → C callus line). Another sizeable number of salt adapted calli for four successive subcultures were shifted to salt free medium and then once again to NaCl supplemented medium (adapted → C → S callus line).

i) Protein banding pattern: Two hundred milligrams of callus was frozen overnight in 1 ml of 0.1 M sodium phosphate buffer (pH 7.0) containing 50 mg polyvinyl pyrrolidine at -4° C and used for extraction of soluble proteins. Protein extracts were used for quantitative estimation by Bradford (1976). Electrophoresis for protein banding pattern was carried out under denaturing conditions following the SDS-PAGE procedure described by Laemmli (1970). The protein extract containing 50 µg proteins was transferred to equal volume of sample buffer, heated at 100°C for three minutes, cooled and used for SDS-PAGE. After cooling the samples were loaded in each well with marker proteins (29-205 kDa range) in a separate well. At the end of electrophoresis, gels were stained with Coomassie blue G-250 and destained until the removal of extra stain. R_f values of standard proteins plotted against $\log_{10}$ of their MW provided a plot for calculating MW of different protein bands.

ii) DNA extraction, amplification and electrophoresis: The protocol described by Williams *et al*. (1990) for PCR was employed as modified by Dhawan *et al*. (2006). Two hundred mg of callus from each treatment was taken and DNA extracted using a kit by Bangalore Genei, Bangalore. For RAPD analysis, DNA was amplified against random decamer primers obtained from Genetix, New Delhi. *Taq* polymerase, dNTPs and 10x assay buffer were obtained from Bangalore Genei, Bangalore.

Reaction mixture (25 µl) for PCR amplification contained 19.0 µl double distilled sterilized water, 2.5 µl 10X assay buffer with 15 mM $MgCl_2$, 1.0 µl primer (50 to 60 ng/µl), 1.0 µl mix of dNTPs (2.5 mM each), 0.5 µl *Taq* polymerase (1.5 unit) and 1.0 µl sample DNA (50 ng/µl). Mixture was spun for 5 sec and kept in the thermocycler (MJ Research, USA) using the following programme: initial denaturation at 94°C for 6 min; denaturation at 94°C for 1 min; annealing at 42°C for 2 min; extension at 42°C for 2 min and final extension at 72°C for 5 min. Forty cycles of denaturation, annealing and extension steps were used.

DNA fragments generated by amplification were separated on 1.5% agarose gel by horizontal electrophoresis, in 1x TAE buffer. PCR products were viewed under UV light using UV Transilluminator

(BIORAD, USA). Molecular weight of different fragments was determined by using 100 base pair ladder run on the gel along with amplification products.

RESULTS

Growth of actively growing callus pieces was inhibited by NaCl; 170 and 190 mM NaCl inhibited callus growth completely. Calluses grown in NaCl free medium and those supplemented with 65 and 85 mM NaCl were light green and fragile while higher NaCl concentrations changed color from light green to light brown and finally brown. The texture of the callus also changed from fragile to compact and hard (Plate 1&2).

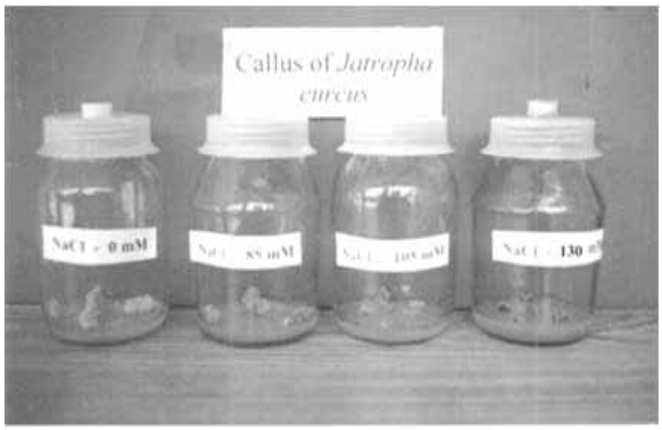

Plate 1. Effect of different concentrations of NaCl on callus growth during second subculture in *J. curcus*

Plate 2. Callus growth in *J. curcus* supplemented with 105 mM NaCl for four generation (on extreme right) and subsequent shifting to salt free medium (Adapted→C, centre)

SDS-PAGE protein profile: Protein profile studies revealed only two proteins of MW 177.8 and 125.9 kDa in control callus line. Out of these, protein of MW 177.8 kDa was uniformly present in all salt treated callus lines. In addition to this eight other proteins were formed in different salt treated calli lines. Unadapted callus line possessed four additional protein bands of MW 100.0, 79.4, 67.6 and 57.5 kDa over control. On the other hand, comparison of unadapted and salt adapted callus line revealed five common protein bands of MW 177.8, 100.0, 79.4, 67.6, 57.5 kDa while three additional protein bands of size 74.1, 50.1 and 39.8 kDa were present in the salt adapted callus line only. Both adapted→ C and adapted→C→S callus lines showed expression of four similar protein bands of MW 177.8, 100.0, 79.4 and 67.6 kDa while an additional band of MW 53.7 kDa was expressed in the adapted→C→S callus line only (Table 1).

DNA amplification: DNA amplification pattern of all callus lines of *Jatropha curcus* was studied with different primers (P1-P10). All ten decanucleotide primers of random sequence used in the present study caused DNA amplification. A total of 78 DNA amplification products were formed against the ten primers (Table 2). Primer P2 amplified the highest number (16) of products followed by primer P1 (13) while against primer P9 only three products were amplified in *J. curcus* callus lines. DNA amplification by different primers is presented in Table 2 and summarized below:

Primer BG52SI10A11: Against this primer, a total of 13 products of 100-1300 bp size were amplified, of which two of size 900 and 450 bp were commonly amplified in all callus lines except adapted →C callus line. In control, products with 330 and 100 bp were amplified in addition to those of 900 and 450 bp. The unadapted and salt adapted calli amplified products of size 1300, 1250, 1200, 1150, 980, 800, 450, 340, 330 and 110 bp while adapted→C→S callus line lacked bands of 330 and 100 bp but had two additional bands of size 220 and 130 bp.

Primer BG47SI10G6: A total of 16 RAPD-PCR products were formed against this primer, of which four

Table 1. SDS-PAGE protein profile in different callus lines of *Jatropha curcus.*

Size of protein (kDa)	Control	Unadapted salt stressed	Adapted	Adapted→C	Adapted→C→S
177.8	+	+	+	+	+
125.9	+	-	-	-	-
100.0	-	+	+	+	+
79.4	-	+	+	+	+
74.1	-	-	+	-	-
67.6	-	+	+	+	+
57.5	-	+	+	-	-
53.7	-	-	-	-	+
50.1	-	-	+	-	-
39.8	-	-	+	-	-

+ or – indicate presence or absence of a particular protein band, respectively.

products of size 1050, 1000, 850 and 180 bp were commonly amplified in all callus lines. Three additional products viz. 780, 500 and 450 bp were amplified in the control. Salt induced nine additional products (1350, 1320, 1270, 1250, 1180, 700, 680, 600 and 400 bp). Unadapted salt stressed callus revealed three distinct products (1320, 1180 and 600 bp) over control. Adapted calli showed six additional RAPD-PCR products (1350, 1270, 1250, 700, 680 and 400 bp) when compared to unadapted salt stressed callus. Adapted→C callus line revealed loss of product size of 700, 680 and 400 bp. The adapted→C→S callus line showed amplification of two additional products of size 500 and 600 bp when compared to adapted→C callus line.

Primer BG42SI10A1: Against this primer, a total of 8 RAPD-PCR products were amplified, out of which products of size 1100 and 1000 bp were common to all callus lines. Three additional products of size 800, 500 and 300 bp were amplified in the control. On the otherhand, salt treated callus possessed three distinct products (1120, 510 and 320 bp). Uunadapted salt stressed calli lacked 800 bp bands over control. However, adapted callus lines amplified products exactly similar to the control with this primer. Comparison of adapted→C and adapted→C→S callus revealed that the former showed amplification of a distinct product of size 1120 bp.

Primer BG53SI10A12: A total of 8 RAPD-PCR products were produced against this primer. Salt treated callus lines had four additional products of size (1020, 820, 450 and 320 bp) over control. Unadapted salt stressed callus lines revealed amplification of two distinct products of size 520 and 480 bp over control. Comparison of salt adapted callus with unadapted ones showed disappearance of a distinct product of size 1020 bp in the former. DNA from adapted→C and adapted→C→S callus amplified similar products viz. 450 and 320 bp.

Primer BG45SI10A4: Against this primer, 8 RAPD-PCR products were obtained. It amplified only two products of the size 1250 and 610 bp in the control callus line. Unadapted salt stressed callus line evinced three distinct bands of size 1000, 760 and 450 bp over control while adapted ones had one unique band of 780 bp but lacked product of 1000, 760 and 450 bp altogether. Adapted→C callus line revealed presence of only one product of size 450 bp while adapted→C→S showed four products (1250, 900, 800 and 610 bp).

Table 2: Amplification products formed in PCR reaction using ten random primers in different callus lines in *Jatropha curcus*.

Primer and sequence (5'-3')	Total products formed and their size (bp)	Product size amplified in different callus lines (bp)				
		Control	Unadapted salt stressed	Adapted	Adapted→C	Adapted→C→S
BG52S110A11 **(P1)** 5'-TGAGCGGACA-3'	1300, 1250, 1200, 1150, 980, 900, 800, 450, 340, 330, 220, 130, 100	900, 450, 330, 100	All except 220, 130	All except 220, 130	—-	All except 330, 100
BG47S110G6 **(P2)** 5'-TGTAGCTGGG-3'	1350, 1320, 1270, 1250, 1180, 1050, 1000, 850, 780, 700, 680, 600, 500, 450, 400, 180	1050, 1000, 850, 780, 500, 450, 180	1320, 1180, 1050, 1000, 850, 780, 600, 500, 180	All except 780, 600, 500, 450	All except 780, 700, 680, 600, 500, 450, 400	All except 780, 700, 680, 450, 400
BG42S110A1 **(P3)** 5'-GAGCCCTCCA-3'	1120, 1100, 1000, 800, 510, 500, 320, 300	1100, 1000, 800, 500, 300	1100, 1000, 500, 300	1100, 1000, 800, 500, 300	1120, 1100, 1000, 800, 510, 320	1100, 1000, 800, 510, 320
BG53S110A12 **(P4)** 5'-GGTGCGGGAA-3'	1250, 1020, 850, 820, 520, 480, 450, 320	1250, 850, 520, 480	1020, 820, 520, 480	820, 520, 480	450, 320	450, 320
BG45S110A4 **(P5)** 5'-TCGGACGTGA-3'	1250, 1000, 900, 800, 780, 760, 610, 450	1250, 610	1250, 1000, 760, 610, 450	1250, 780, 610	450	1250, 900, 800, 610
100CO4 **(P6)** 5'-CCGCATCTAC-3'	1100, 1050, 820, 780, 510	780	1100, 1050, 780	1150, 1050, 820, 780, 510	820, 780, 510	1100, 1050, 820, 780
90B11 **(P7)** 5'-GTAGACCCGT-3'	1020, 1000, 700, 320	700	1020, 700, 320	1020, 700, 320	1020, 700, 320	1020, 1000, 700, 320
60BO4 **(P8)** 5'-GGACTGGAGT-3'	1100, 800, 530, 400, 320	—-	100, 800, 530, 320	All	——-	530, 400
BG49S110G8 **(P9)** 5'-AATGGCGCAG-3'	1100, 1010, 340	All	All	All	All	1100
BG56S110C15 **(P10)** 5'-CCCAAGGTCC-3'	1300, 1200, 950, 810, 780, 520, 400, 280	All except 1300, 400	All	All	All except 400	All except 400

Primer 100CO4: Against this primer, only five RAPD-PCR products were formed, amongst which a product of 780 bp size was common in all the callus lines and this was the only product amplified in the control callus line. Unadapted salt stressed callus lines had two additional products of 1100 and 1050 bp size over control while adapted callus lines revealed presence of two more bands of 820 and 510 bp. Adapted→C callus line showed presence of additional band 780 bp while adapted→C→S revealed amplification product of size 1100 and 1050 bp.

Primer 90B11: A total of 4 RAPD-PCR products were amplified by this primer, out of which a product of 700 bp size was common to all callus lines and this was the only product amplified in the control callus line. Unadapted salt stressed callus, adapted and adapted→C callus lines had two additional products of size 1020 and 320 bp over control. One distinct band of size 1000 bp appeared in adapted→CS callus which was not formed in any other callus line.

Primer 60BO4: Against this primer, 5 products (1100, 800, 530, 400 and 320 bp) were amplified. No amplified product was obtained in control as well as adapted → C callus lines. Salt treatment of callus induced product of size 530 bp except the adapted→C callus line. Unadapted salt stressed callus had four amplified products (1100, 800, 530 and 320 bp). Adapted callus line on the other hand, produced all the five bands while adapted→C→S callus line amplified product of the size 530 and 400 bp only.

Primer BG49SI10G8: Against this primer, only 3 products (1100, 1010 and 340 bp) were formed. A product of size 1100 bp was amplified in all callus lines. Control, unadapted, salt adapted and adapted→C callus lines had same RAPD-PCR products. Adapted→C→S callus line produced band of 1100 bp only.

Primer BG56SI10C15: A total of eight products were amplified by this primer, out of which six products of size (1200, 950, 810, 780, 520 and 280bp) were amplified in all callus lines. Unadapted and salt adapted callus lines had two additional products of size 1300 and 400bp over control while in adapted→C and adapted→C→S callus lines product of size 400 bp was missing.

DISCUSSION

SDS-PAGE protein profile of control callus line revealed only two polypeptide bands of MW 177.8 and 125.9 kDa. Sudden exposure of callus to NaCl induced synthesis of altogether a new set of four polypeptides with MW 100, 79.4, 67.6 and 57.5 kDa which can be designated as 'salt responsive polypeptides'. Repeated sub-culture of callus on salt supplemented medium induced biosynthesis of additional polypeptides with MW 177.8, 74.1, 50.1 and 39.8 kDa but lacked polypeptide of 67.6 kDa. Shifting of salt adapted callus line to salt free medium (adapted→C) retained biosynthesis of polypeptides with MW 177.8, 100, 79.4 and 67.6 kDa while all other bands disappeared. Re-shifting of these calli to the salt supplemented medium (adapted→C→S) induced synthesis of a new polypeptide of MW 53.7 kDa. These results vividly evince that in *J. curcus* polypeptides with MW 100, 79.4 and 67.6 kDa are always expressed upon exposure of callus to NaCl which seem to be critical to its survival while all other bands were transiently expressed. Ali *et al*. (1999) reported that NaCl induced expression of bands of approx. 58-60, 62-64 and 66-68 kDa as compared to control in regenerants of *Brassica monniera*. Higher concentrations of NaCl induced expression of new polypeptide bands of ~88-90, ~92-94 and ~104-106 kDa. Similarly, El-Baky *et al*. (2003) found that although salt stress induced three new protein bands with MW of 50, 66 and 80 kDa in three cultivars of onion, band of 66 kDa was the most common in all treatments. Although Pareek *et al*. (1997) suggested that stress proteins could be used as important molecular markers for improvement of salt tolerance using genetic engineering techniques; in many studies the proteins produced under salt stress are not always associated with salt tolerance thereby indicating that utility of proteins as salt tolerance marker depends on the plant species or cultivar.

All the ten decanucleotide primers of random sequence used in the present study caused DNA amplification. A total of 78 DNA amplification products were formed. Primer P2(5'-TGTAGCTGGG-3') amplified the highest number (16) of products followed by primer P1 (5'-TGAGCGGACA-3',13) while against primer P9 (5'-AATGGCGCAG-3') only three products of size 1100, 1010 and 340 bp were amplified

irrespective of the callus source thereby indicating that no distinct product was amplified in response to salt stress. Against primer P4 (5'-GGTGCGGGAA-3'), DNA of unadapted salt stressed callus line amplified only two products of size 1020 and 820 bp. Among these, product of size 1020 bp disappeared in the salt adapted callus line indicating this product to be a transient one. None of these products were amplified in adapted→C and adapted→C→S callus lines. Rather two distinct products of size 450 and 320 bp were amplified.

Against primer P6 (5'- CCGCATCTAC -3'), DNA of unadapted and salt adapted callus lines revealed amplification of common products of size 1100 and 1050 bp while salt adapted one showed two additional products of size 820 and 510 bp. In adapted→C callus line amplification of products of size 820 and 510 was evident while adapted→C→S showed amplification of products of size 1100 and 1050 bp. This indicates that products of size 1100 and 1050 bp are amplified as initial response products, while that of 820 bp is amplified in the salt adapted callus lines. Similarly, against primer P8 (5'-GGACTGGAGT-3'), DNA of unadapted callus line amplified four products of size 1100, 800, 530 and 320 bp while that of salt adapted callus line had an additional amplification product of size 400 bp. DNA of adapted→C→S callus line amplified products of size 530 and 400 bp only. It evinces that products of 1100, 800 and 320 bp are expressed transiently as initial response product while those of size 530 and 400 bp are expressed in salt adapted callus line.

Against primer P7 (5'- GTAGACCCGT-3'), DNA of unadapted , salt adapted and adapted→C callus lines amplified products of size 1020 and 320 bp while one distinct product of size 1000 bp appeared in adapted→C→S callus line. It suggests that induction of products of size 1020 and 320 is persistent. RAPD-PCR of DNA of unadapted and salt adapted callus lines amplified two distinct products of size 1300 and 400 bp against primer P10 (5'-CCCAAGGTCC-3'). This primer amplified the product of size 1300 bp only in the adapted→C and adapted →C→S callus lines that suggests salt stress induced amplification of two products of size 1300 and 400 bp but the latter was transient in its expression.

It may be deduced from RAPD-PCR of DNA callus lines that some of the products were amplified consistently upon exposure to salt stress (1020, 820 and 510 bp against primer P6; 320 bp against primer P7 and 400 bp against primer P8) while others were expressed transiently (1020 bp against primer P4 and 1400 bp against primer P10). Dhawan *et al*. (2003) reported that some RAPD fragments were specifically produced in freeze tolerant (RH-781, RH-8814) or susceptible (RH-30) genotypes of *Brassica juncea* and suggested that primer 5'-GTGATCGCAG-3' and 5'-TCGGCGATGA-3' could be used as freeze tolerance markers in *Brassica* genotypes. Weng and Chen (2002) identified primer OPZ09 which was polymorphic with a fragment of 590 bp in two parents of wheat (Nongda 8502-female, salt sensitive and Chadian Red- male, salt tolerant) and F_1 and F_2 populations so the specific fragment of OPZ09-590 was a RAPD marker linked to salt tolerance gene in Chadian Red. Xie *et al.* (2000) compared the genetic diversity of salt tolerant rice varieties (Pokalli, Bicol) and salt sensitive variety (IR29) using RAPD markers. Four primers –amplified specific fragments appeared in all the salt tolerant varieties but not in the salt susceptible variety.

Thus, polypeptides with MW 100.0, 79.4 and 67.6 kDa synthesized in response to NaCl seem to be critical for survival under salt stress. Primers like 100C 04(5'-CCGCATCTAC-3'), 90B11 (5'-GTAGACCCGT-3'), and BG56SI10C15 (5'-CCCAAGGTCC-3') can be employed to detect amplification products related to salt tolerance in *J. curcus*.

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