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Selection and characterization of salt tolerant callus culture and *In vitro* regeneration in chickpea (*Cicer arietinum* L)

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Abstract
Callus cultures of chickpea (*Cicer arietinum* L.) were established from mature embryos of chickpea. The callus were subcultured on the medium with different concentration of NaCl for three passages. Callus growth as fresh weight, fold increase and percent inhibition of all chickpea genotypes was generally depressed as salt concentration increase in the culture medium. The results clearly showed that, there is an increase in the amount of sodium content taken by the different chickpea calli which parallels the increase salt level in the culture media. Whereas, there was a slight decrease in postassium content with increasing salt levels in the culture media.

Keywords: salt tolerant callus, regeneration in chickpea (*Cicer arietinum* L)

Introduction
Chickpea (*Cicer arietinum L.*) is an important grain legumes which has worldwide acceptance as a major source of protein for human as well as animal consumption. Some of the major constraints identified in the production of this crop are (1). Low adaptability and stability of yield (2). Tendency for excessive vegetative growth under irrigation and other inputs and (3) susceptibility to stresses like salinity, disease and insect pest (Jaiswal, R and Singh NP, 2001) [5]. The use of tissue culture techniques has the potential to increase the stress tolerance of plants because the plant cells contain a complete species genomic and thus are totipotent.

Materials and Methods
Preparation of Plant Material and Selection Procedure of Culture
Seeds of chickpeas genotypes *viz*. K 850, CSG 8962 Kernal chana-1, Bio 102 and CSG 8890 were secured from Genetic Resource Unit, IIPR, Kanpur. Each genotypes were surface sterilized with three drops of liquid detergent (tween 20) and the immersed in 70% ethanol for 2 minutes followed by 10% sodium hypochlorite solution for 5 minutes then seeds were washed with double distilled water for four times. Seeds were soaked in a sterile distilled water overnight. A stock culture was established by subcultured of callus tissue to fresh media at 20 days of intervals. For selection procedure, callus tissue (0.5 gm) of the different cultivars were grown on Mature embryos of chickpea genotypes were aseptically dissected out and placed on MS medium supplemented with 0.5 mg/l, NAA+ 0.5 mg/l, BAP+40 g/l, sucrose and 0.8% agar for callus induction. The calli produced were further subcultured on the same medium enriched with different concentrations (0%, 0.25%, 0.5% and 1.0%) of NaCl. Callus from each explant was maintained separately and screened against different doses of NaCl. The embryogenic calli that were multiplying/growing on stress medium were selected for three cycles at intervals of 20 days. Such selected callus pieces were designated as variant/tolerant. The growing calli were further transferred to regeneration medium without NaCl stress (MS salts+B5 vitamins+0.125 mg/l IBA+2.0 mg/l BAP+ 40 g/l sucrose+0.8 % agar). Tolerant regenerants were further subjected to stability test in NaCl stress medium for confirmation.
The stability of resistant clones was estimated by calculating the number of "escapes" in selected population as follows:

Number of escapes= (b-c)/aX100

Where a=total number of explants, b= number of selected resistant clone(s) and c= stable clone(s).
Na+/K+ ion uptake of selected and non-selected calli/cell lines is depicted in table 3. The growth of the callus was highly affected by the concentration of NaCl. In general, C2 callus/cell lines showed higher uptake of Na+/K+ than Cl- callus/cell lines. Concentration of the K+ ion content was significantly higher in selected callus lines than the non-selected lines. There was no significant difference among genotypes with regard to Na+/K+ ratio indicating no significant difference in tolerance levels to NaCl. The findings can agree with the report of Sanagavan *et al.*

Result and Discussion
Table 1: Selection and survival of chickpea calli and regenerants against sodium chloride (NaCl)

Cycle	Days	0%NaCl		0.25% NaCl		0.5% NaCl		1.0%NaCl	
		No.	%	No.	%	No.	%	No.	%
I	20	298	100	244	100.0	244	100.0	236	100.0

II	40	296	99.3	152	62.3	148	60.6	78	33.1
III	60	295	98.9	149	61.1	130	53.3	50	21.2

Table 2: Recovery of resistant clones of chickpea in medium with different concentration of sodium chloride (NaCl)

Concentration of NaCl (%)	No. of explants (a)	Resistant Clones				
		Selected		Stable		No. of escapes
		No. (b)	%	No. (c)	%	(b-c/a)X100
0(control)	295	290	98.3	265	89.9	08.5
0.25	149	90	60.4	75	50.3	10.1
0.5	130	75	57.7	65	50.0	07.7
1.0	50	20	40.0	08	16.0	24.0

Table 3: Na+/K+ uptake in callus growth *in vitro* culture of NaCl: CaCl2

Salt Concentration (ppm)	Uptake of Na+(ppm)		K+		K+/Na+ ratio			
	C1	C2	C1	C2	C1	C	2	
Control	170	180	070	080	412	44	4	+9.63

The effect of NaCl on callus was observed as changes in colour (Browning) and texture (Compactness). The magnitude of callus growth was dependent on the concentration of NaCl and incubation period of cultures in presence of NaCl. The control (no stress) resulted in green and friable callus, while the NaCl treatments showed varied degree of browning and necrosis depending on the concentration of NaCl used.

The recovery of salt tolerant/ adapted calli decreased with increase in concentration of NaCl. The lowest recovery (21.2%) of adapted calli was observed on 1.0% NaCl, whereas maximum recovery (61.1%) was obtained on medium containing 0.25% NaCl (Table 1). In general, a substantial reduction in number of selected clones was observed after subsequent cycle of selection (direct stepwise). Further it was observed that frequency of stable resistant clones declined substantially on increasing the dose of NaCl. Highest frequency (50.3%) of stable clones were obtained at 0.25% NaCl concentration and lowest frequency (16.0%) at 1.0% NaCl (Table 2). Pandey and Ganapathy (1984) [8] and Gosal and Bajaj (1984) [4] also isolated salt tolerant cell lines of chickpea. But, they failed to regenerate the salt tolerant cell lines. Further, Singh *et al*. (1999) [11] reported, regeneration of Aschochyta blight resistant cell lines in chickpea. However, the progenies of these salt tolerant plantlets need to be analyzed in order to confirm the genetic basis of salt tolerant trait.

Na+/K+ ion uptake of selected and non-selected calli/cell lines is depicted in table 3. The growth of the callus was highly affected by the concentration of added NaCl. In general, C2 callus/cell lines showed higher uptake of Na+/K+ than C1- callus/cell lines except in few levels of salt and salt mixtures. The K+ ion content were significantly higher in selected callus lines than the non-selected callus lines. There was no significant difference among genotypes with regard to Na+/K+ ratio indicating no significant difference in tolerance levels to NaCl (data not shown). The findings can agree with the report of Sanagavan *et al*. (1997) [10].

Many investigator have been studied the effect of salinity stress on callus growth of chickpea extent of change in ploidy level of calli cells resulting in disappearing of genes associated to regeneration ability. The report by Chandler and Vasil (1984) [3] revealed that the presence of salt in the culture medium may be inhibitory to regeneration especially by somatic embryogenesis of cell lines of *Pennisetum purpurenum*. In this connection, it should be mentioned that, plants have been regenerated from NaCl-tolerant cells of oats (Nabors *et al* 1982) [7], rice (Reddy *et al* 1986) [9], napier grass (Chandler SF 1984) [3] and Sorghum (Bhaskaran *et al*, 1986) [2], Chickpea (Timothy J. Flowers *et al*, 2010) [12]. The some of these reports, tolerance to NaCl was expressed in a few of the regenerated plants but as yet there is no tolerant plants from tissue cultures of the pulses. These disappointing results may possibly be a consequences of the number of factors, including (1) the polygenic nature of salt tolerance and (2) the use of NaCl alone as a stress rather than in combination with other ions, as may occur in natural environment or *in vitro* anomalies, including the physiological adaptation of culture cells, (4) the non-regeneration of selected cell types, (5) the phenotypic changes in regenerated plants and (6) the developmental regulation of cellular expression of salt tolerance (Morrish *et al*. 1987).

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