EFFECT OF NaCl STRESS ON CALLUS MORPHOLOGY AND GROWTH OF SUGARCANE CALLUS CULTURES (cv. SPF 234 AND cv. HSF 240)

N. Munir[*] and F. Aftab[**]

[*]Department of Biotechnology, Lahore College for Women University, Lahore
[**]Department of Botany, University of the Punjab, Q. A. Campus Lahore-54590, Pakistan.
Corresponding Author E-mail: neelma.munir@yahoo.com

ABSTRACT: Salt stress is one of the major abiotic stresses limiting crop productivity. Growth of sugarcane which is an important cash crop is affected by various biotic and abiotic stresses. As sugarcane is a glycophyte it do not thrive well in saline soils. Response of two sugarcane cultivars (cv. SPF 234 and cv. HSF 240) was observed in response to various salt levels during the present study. Callus cultures were transferred to the standardized media for callus formation supplemented with various NaCl concentrations (MS + 13.5 µM 2, 4-D + 0-160 mM NaCl; 9 treatments). A variation in callus morphology was observed in both the tested sugarcane cultivars upon transfer to NaCl-containing media. It was found that the callus cultures become brown and necrotic when subjected to salt stress. A general decrease was observed in fresh weights of callus cultures when subjected to salt stress. However less fresh weight reduction was recorded in SPF 234 callus cultures as compared to callus cultures of cv. HSF 240. Hence SPF 234 appeared to be more salt tolerant as compared to cv. HSF 240 under *in vitro* conditions. This study therefore, is an important step forward in improving salt tolerance of sugarcane by *in vitro* techniques.

INTRODUCTION

Soil salinity is a major problem that threatens the productivity of crops in many countries of the world including Pakistan (Ashraf, 1994). It has been reported that 397 million hectares of land throughout the world are salt affected (FAO, 2005). Sugarcane is an important industrial cash crop in Pakistan but its annual yield is far below the existing potential. Soil salinity is one of the reasons that result in low productivity of sugarcane (Akhtar *et al*., 2003). Furthermore, it also decreases the quality of sugarcane juice by decreasing its sucrose content (Lingle and Weigand, 1996). Salt stress is expressed by many morphological and metabolic changes

Plant tissue culture techniques provide a useful tool to produce stress tolerant plants including the plants resistant to soil salinity. These techniques provide a promising and feasible approach to develop salt tolerant plants (Jain, 2001). Not much work has been reported on *in vitro* selection and response of callus cultures towards salt stress in Sugarcane cultivars growing in Pakistan. The present investigation was conducted to observe the *in vitro* response of two sugarcane cultivars (cv. SPF 234 and cv. HSF 240) towards NaCl stress.

MATERIALS AND METHODS

Establishment of Callus cultures: Callus cultures were established from explants ranging from 5-8 mm in diameter (2-3 mm thick) derived from young inner 2-3 whorls of leaves of two sugarcane cultivars (SPF 234 and HSF 240) on MS (Murashige and Skoog, 1962) medium supplemented with 13.5 µM 2,4-D. The young inner 2-3 whorls of sugarcane leaves, wrapped deeply within the mature ones were used as explants. The inner leaves were surrounded by many layers of leaves and therefore, did not require any particular disinfection. However, to minimize the chances of contamination, during explant preparation, 20-30 cm long field-grown plant material was initially surface sterilized with 95 % ethyl alcohol

The calluses were induced under dark conditions and later transferred to 16-h photoperiod (35 µmol m^{-2} s^{-1}) provided by cool fluorescent tube lights 27 ± 2 ºC

Salt stress to callus cultures: Different concentrations of NaCl were used in the present study and added directly into already prepared MS medium. Medium was supplemented with 9 different salt concentrations (0-160 mM) to observe the effect of salt stress on sugarcane callus culturesCallus morphology and fresh weights (g) were recorded at day 90, 120 and 150.

Experimental Design: The experiment was perfomed in complete randamized design with 20 replicates per treatment. Each experiment was repeated thrice.

Statistical Analysis: The data was analyzed by univariate analysis of variance by using SPSS version 11. The values were also compared statistically by Duncan's multiple range test.

RESULTS

During the present study, it was observed that sugarcane callus cultures underwent several

morphological changes with the addition of salt in the medium over successive subcultures. Morphology of sugarcane callus cultures (cvs. SPF 234 and HSF 240) at various NaCl levels is shown in Table 1 and 2, respectively. It is evident from the data that 60-days-old callus cultures of cv. SPF 234 maintained on MS basal medium supplemented with 13.5 μM 2,4-D were greenish-yellow, friable and granular. When 60-days-old calluses were shifted to the same medium containing various NaCl levels (0-160 mM), variation in callus morphology of both the cultivars was observed. It was observed that calluses during different subcultures (at day 90, 120 or 150) had no signs of necrosis in the absence of salt in the medium. However on NaCl containing medium (20-160 mM NaCl) callus cultures turned brown and complete necrosis of callus culture was obtained at 160 mM NaCl at day 90 and further maintenance was not possible due to necrosis.

For cv. HSF 240, the callus color and morphology was also found to be affected with increasing salt concentration. The callus cultures before NaCl treatment at day 60 were greenish-yellow and friable. Like the other cultivar, most of the callus cultures of cv. HSF 240 maintained at 0 mM NaCl level did not show any sign of necrosis during different subcultures at day 90, 120 or 150. Upon shifting of these callus cultures to MS medium with various salt concentrations, changes in morphological characteristics were recorded. It was observed that with an increase in salt concentration as well as with the passage of time (during successive subcultures), greenish-yellow callus started turning brownish in color. For this cultivar (unlike SPF 234), complete browning of the callus cultures, however, was observed when MS medium was supplemented with 140 mM NaCl level or above. Representative pictures of callus cultures at 0mM NaCl level and at various salt levels are given in Fig 1.

A significant effect of the tested media was recorded on fresh weights of callus cultures at day 90, 120 and 150 after NaCl treatment (Table 3). It was observed that callus cultures of both the cultivars showed a gradual decrease in fresh weights with an increase in salt concentration at day 90, 120 or 150 as compared to the control. During different subcultures, fresh weights of the calluses of both sugarcane cultivars maintained at 0 mM NaCl level (control), increased slightly up to day 150, i.e., the final day of data collection. The callus cultures of cv. SPF 234 at day 120 up to 60 mM NaCl level had slightly greater fresh weights as compared to callus cultures maintained on the same NaCl level at day 90. At day 150, once again reduction was observed in fresh weights of callus cultures subjected to various NaCl concentrations. Almost similar growth trend was observed in callus cultures of cv. HSF 240. However, at 40 or 60 mM NaCl levels, fresh weight of callus cultures at day 120 had similar values (1.06 g). For this cultivar, once again reduction was observed in fresh weights of NaCl-treated callus cultures as compared to the control at day 150. The overall significance thus depicts reduced callus growth with an increasing salt level.

Table 1: Morphology of sugarcane callus cultures (cv. SPF 234) at different NaCl levels (0-160 mM) supplemented to MS medium under dark conditions at day 90, 120 and 150 [a]

NaCl (mM)	Callus morphology before NaCl treatment	Morphology of sugarcane callus cultures after NaCl treatment		
	At day 60	At day 90	At day 120	At day 150
0	Greenish-yellow, friable, granular	Greenish-yellow, friable, granular	Greenish-yellow, friable, granular	Whitish-yellow, friable, granular
20	Greenish-yellow, friable, granular	Greenish-yellow, friable, granular	Greenish-yellow with some whitish portion, granular	Brownish-yellow, friable, granular
40	Greenish-yellow, friable, granular	Brownish-yellow, friable, granular	Greenish-yellow, friable, granular	Greenish-brown, smooth surfaced
60	Greenish-yellow, friable, granular	Greenish-yellow granular	Greenish brown, friable, granular	Yellowish-brown, friable,granular
80	Greenish-yellow, friable, granular	Off-white with some brown portion, granular	Off-white with some reddish- brown portion, granular	Blackish-brown, friable, granular
100	Greenish-yellow, friable, granular	Brownish-off white, friable, granular	Yellowish-brown, friable, granular	Yellowish-brown, translucent
120	Greenish-yellow, friable, granular	Brownish-off white, friable, granular	Blackish-brown, necrotic	Blackish-brown, necrotic
140	Greenish-yellow, friable, granular	Brownish-off white, friable, granular	Blackish-brown	Brownish-black, necrotic
160	Greenish-yellow, friable, granular	Brownish-black	ND [b]	ND [b]

[a] Callus morphology is based on 60 culture vessels per NaCl treatment.
[b] ND: Not determined because callus cultures could not be maintained at these salt levels over successive subcultures

Table 2: Morphology of sugarcane callus cultures (cv. HSF 240) at different NaCl levels (0-160 mM) supplemented to MS medium under dark conditions at day 90, 120 and 150 [a]

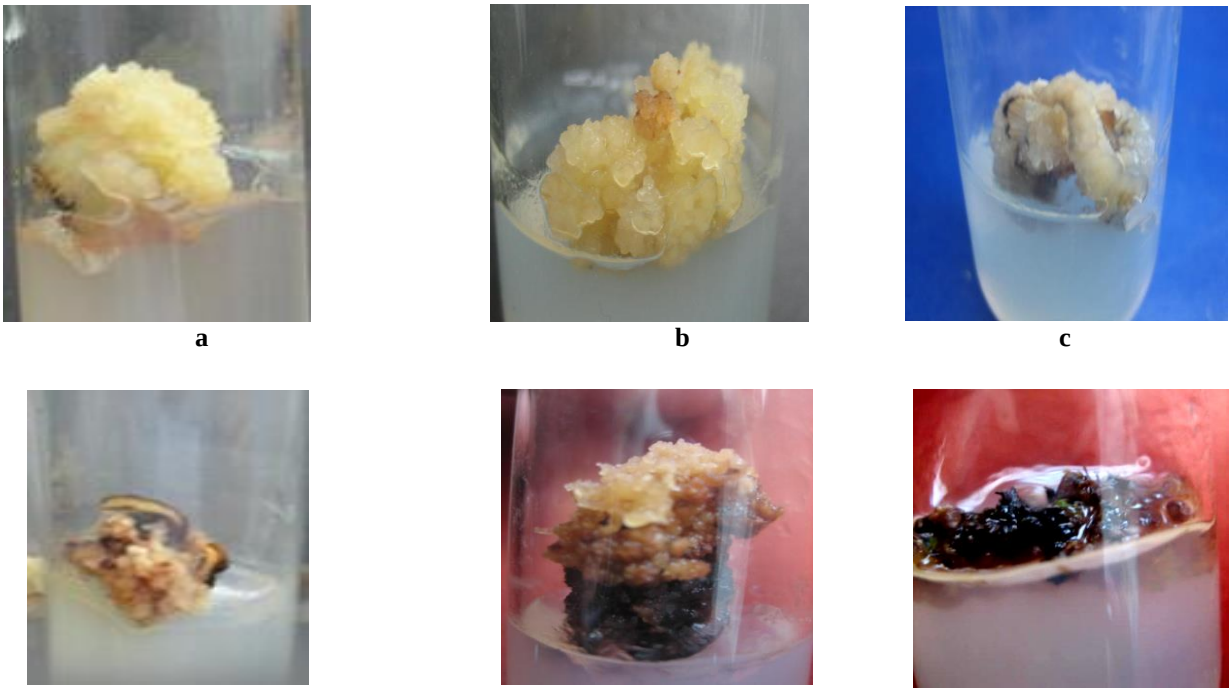
NaCl (mM)	Callus morphology before NaCl treatment	Morphology of sugarcane callus cultures after NaCl treatment		
	At day 90	At day 90	At day 120	At day 150
0	Greenish-yellow, friable, granular	Greenish-yellow, friable, granular	Greenish-yellow, friable, granular	Translucent, yellowish-green
20	Greenish-yellow, friable, granular	Greenish-yellow, friable, granular	Off white with some brown portion, friable, granular	Yellowish-brown, friable, granular
40	Greenish-yellow, friable, granular	Brownish-yellow, friable, granular	Greenish-brown, friable, granular	Greenish-brown, smooth
60	Greenish-yellow, friable, granular	Brownish-yellow, friable, granular	Brownish off white, friable, granular	Yellowish-brown, smooth
80	Greenish-yellow, friable, granular	Brownish-yellow, friable, granular	Brownish off-white, friable, granular	Brownish with some off-white portion, friable, granular
100	Greenish-yellow, friable, granular	Brownish-off white, translucent	Blackish-brown, friable, granular	Brownish-black, smooth surfaced
120	Greenish-yellow, friable, granular	Brownish off-white, friable, granular	Blackish-brown, necrotic	Brownish-black, necrotic
140	Greenish-yellow, friable, granular	Blackish-brown, necrotic	ND [b]	ND [b]
160	Greenish-yellow, friable, granular	Brownish-black, necrotic	ND [b]	ND [b]

[a] Callus morphology is based on 60 culture vessels per NaCl treatment.
[b] ND: Not determined because callus cultures could not be maintained at these salt levels over successive subcultures.

Table 3: Effect of different NaCl levels (0-160 mM) supplemented to MS medium on fresh weights of sugarcane (cvs. SPF 234 and HSF 240) callus cultures under dark conditions at day 90, 120 and 150

NaCl Level (mM)	Fresh weight of callus cultures [A] (g)					
	Cultivars					
	SPF 234			SPF 234		
	Days			Days		
	90	90	90	90	90	90
0	1.24 [a]	1.29 [a]	1.29 [a]	1.22 [a]	1.26 [a]	1.30 [a]
20	1.14 [b]	1.20 [b]	1.17 [b]	1.12 [b]	1.18 [b]	1.02 [c]
40	1.13 [b]	1.17 [bc]	1.04 [c]	1.13 [b]	1.06 [c]	1.0 [d]
60	1.13 [b]	1.19 [c]	1.05 [c]	1.12 [b]	1.06 [c]	1.0 [d]
80	1.10 [c]	1.07 [d]	1.02 [d]	1.04 [c]	1.07 [c]	1.05 [b]
100	1.04 [d]	1.05 [d]	1.01 [d]	1.01 [cde]	0.99 [d]	0.98 [d]
120	1.04 [d]	1.02 [e]	1.01 [d]	1.02 [cd]	1.02 [cd]	0.98 [d]
140	1.03 [d]	1.02 [e]	0.98 [e]	0.90 [de]	ND [B]	ND [B]
df	**8 and 261**	**7 and 232**	**7 and 232**	**8 and 261**	**6 and 203**	**6 and 203**

[A] Data presented here are the means of 60 culture vessels per NaCl treatment (20 replicates per treatment and the experiment was repeated thrice).
Different letters within a specific column represent significant difference at P= 0.05 according to Duncan's Multiple Range Test.
Data were transformed using $3\sqrt{y}$ (where y is the fresh weight) to normalize the data. Non-transformed mean values are presented.
[B] ND represents that the value could not be determined due to complete callus necrosis.
[*, NS] Significant at 1% level (*) or non-significant (NS) according to F test with df mentioned against each.



a **b** **c**

d **e** **f**

Fig 1: Effect of Salt stress on callus morphology of sugarcane at
a) 20mM NaCl (cv.HSF 240) b) 60mM NaCl (cv.HSF 240) c) 80mM NaCl (cv. SPF 234)
d) 100mM NaCl (cv.HSF 240) e) 140mM NaCl (cv. SPF 234) f) 160mM NaCl (cv. SPF 234)

DISCUSSION

It is evident from the results of this study that 60-days-old callus cultures on MS basal medium were greenish yellow, friable and granular. When 60-days-old calluses were shifted to various NaCl levels (0-140 mM), variation in callus morphology was quite distinct and data to that effect were recorded at day 90, 120 and 150. Thus our results conform to the earlier findings of Gandonou *et al.,* (2005 a, b) who have also reported that salt stress affect callus morphology. It is not surprising to note that these results are also true for many other members of the family Poaceae, e.g., wheat (Karadimova and Dambova**,** 1993). Arzani and Mirodjagh (1999) have also observed similar results and reported brown coloration, necrosis and inhibited growth of callus cultures of wheat at higher NaCl concentrations. Jaiswal and Singh (2001) observed in chick pea that the control treatment (without stress) could establish green and friable callus while NaCl treatments resulted in varying degrees of browning and necrosis depending on the NaCl concentration used.

During the present work, a decrease in fresh weight of callus cultures was also observed with increasing salt concentration in the medium. Decline of callus growth due to NaCl stress as observed in the present study is a usual phenomenon in many plant tissues subjected to stress (Greenway and Munns, 1980; Reddy and Vaidyanath, 1986; Rains, 1989; Cushman *et al*., 1990). This retardation of growth may be due to the fact that certain amount of the total energy available for tissue metabolism is channeled to resist the stress (Cushman *et al*., 1990).

In sugarcane, Gandonou *et al*., (2005 a, b; 2006) and Errabii *et al*., (2006) studied the effect of salt on sugarcane callus cultures and found that fresh weight of calluses decreased with the corresponding increase in the concentration of NaCl in the culture medium**.** A gradual decrease in fresh as well as dry weights under salt stress has also been reported by Rahnama *et al.,* (2003) and Agarwal and Pandey (2004) in potato and cassia plants, respectively. Similarly, Nasir *et al*., (2000) also observed a same decrease in fresh weight of sugarcane seedlings under salt stress.).

It was observed that the two sugarcane cultivars used during the present study (cv. SPF 234 and cv. HSF 240) were different in their salt tolerance level. This was quite evident by the data on fresh weight of callus cultures after salt stress treatments. Similar findings were reported for rice (Lutts *et al.,* 1996; Basu *et al.,* 2002), wheat (Barakat and Abdel-Latif, 1996) and sunflower (Alvarez *et al*., 2003) where NaCl stress was shown to reduce callus growth and the fact that different genotypes responded differently.

In conclusion, results of the study indicate that the different response of cultivars towards salt stress that can be an important step forward in using the cultivars for *in vitro* selection and improving their salt tolerance.

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