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Full Length Research Paper

In vitro performances of hypocotyl and cotyledon explants of tomato cultivars under sodium chloride stress

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A plant tissue culture technique is a good method for the evaluation and screening of plant genotypes for salt tolerance. *In vitro* evaluations of sodium chloride (NaCl) effects on two tomato cultivars (Pearl and Beril) were investigated with four NaCl levels (0, 25, 50 and 75 mM) using hypocotyl and cotyledon explants. The explants were cultured in MS media having 2.0 mg/l BAP along with different concentrations of NaCl. Sodium chloride stress negatively affected the growth traits and chlorophyll content. Significant differences were noticed between the cultivars followed by different NaCl levels, where the Beril responded superior than that of Pearl. The type of explant showed a difference in their response to shoots regeneration under NaCl stress, where the cotyledon explants achieved best results than hypocotyl explants.

Key words: Cotyledons, hypocotyls, *In vitro*, salt stress, tomato.

INTRODUCTION

Tomato is one of the most important vegetable crops in the world. It is considered as the second vegetable crop in the world after potato (Bhatia et al., 2004; Foolad, 2004). The tomato crop is very multipurpose and grown either for fresh market or processing. Tomato is rich in vitamin A, C and fiber and also free from cholesterol (Rao and Agarwal, 2000).

Soil and water irrigation salinity is one of the most environmental problems that threaten agricultural production, especially in arid and semi-arid regions. Increased salinity of soil and irrigation water adversely affect crop yield (Yokoi et al., 2002; Foolad, 2004; Smith et al., 2003). Increase of salinity in agricultural land significantly influenced the production of agricultural crops. United Nations Environmental program estimate that about 20% of agricultural land and 50% of cropland in the world is under the effect of salt stress (Flowers and Yeo, 1995). With the passage of time, and due to climate change and reduced rainfall in arid and semi-arid regions, the use of irrigation water containing a high percentage of salts, in these lands will result in the marginal land becoming unfit for cultivation in the future.

Tissue culture technique provides a unique chance for studying many aspects of plant growth and development (Cano et al., 1998; Shatnawi, 2006). Furthermore, tissue culture gives a good tool for studying the physiological effects of salt at the cellular level under controlled environment (Olmos et al., 1994), and gives useful information to make clear the plant response to salt stress (Shibli et al., 1992). Addition to, offers greater control than *in vivo* growth conditions and has the benefit of small scale with clear visibility for observation shoot and root responses in the presence of enforced stress (Shibli et al ., 1992), as well as the focus on physiological

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Abbreviations: **MS**, Murashige and Skoog; **BAP**, 6-benzylaminopurine.

and biochemical processes, which contribute to salinity tolerance (Rus et al., 1999; Shibli and Aljuboory, 2002). *In vitro* culture of tomato has been successfully exploited for selection of tolerant cell lines for various biotic and abiotic stresses under laboratory conditions, as it requires comparatively less effort and fewer resources than selection of tomato genotypes under field conditions (Bhatia et al., 2004). Different explants were used for study regeneration in tomato and another plant *in vitro* under salt stress example hypocotyl (El-Meleigy et al., 2004; Mohammed et al., 2007; Aazami et al., 2010), cotyledons (El-Meleigy et al., 2004), true leaf (El-Meleigy et al., 2004) and shoot apex (Mohammed et al., 2007). El-Anany (1997) studied shoot regeneration from hypocotyl and cotyledon under salt stress and showed that decrease in shoot number with increase in salinity and high level (100, 150 mM) NaCl inhibited shoot regeneration. On the other hand, Mohammed et al. (2007) used shoot apices explants and found the shoot length decreased with increase in salinity. Abed-Alrahman et al., (2005) and Fayek et al. (2010) respectively showed decrease in growth traits of cucumber (*Cucumis sativus* L.) and Jojoba (*Simmondsia chinensis*) with increased salinity *in vitro*. Potluri and Prasad (1994) used the auxiliary bud in potato cultivars and found that salt stress decreased the shoot length, dry matter and number of shoots. The objective of this study was to find out the effect of NaCl at varied levels on growth and chlorophyll content of two tomato cultivars by using hypocotyls and cotyledons explants.

MATERIALS AND METHODS

Two tomato cultivars of Pearl and Beril were used in this study and their seeds were collected from local companies in Malaysia.

Sterilization and germination of seeds

The collected seeds were subjected for sterilization followed by 8% Clorox (sodium hypochlorite) for 10 min soaked and washed at three times by double distilled water. The seeds were cultured in MS basal medium (Murashige and Skoog, 1962) and incubated in the dark for four days; thereafter it was transferred to the growth room and maintained 16/8 light/ dark condition for ten days.

After two weeks of germination *in vitro*, the hypocotyls and cotyledons explants were excised (approximately 1 cm) and cultured in MS media supplemented with 2.0 mg/l BAP, 3% sucrose, 0.7% agar and added with a range of NaCl (0, 25, 50 and 75 mM) treatments. The 50 ml of medium was dispensed in each of the culture vessels. The pH of the media was adjusted to 5.8±1 and autoclaved at 121°C for 20 min. The cultures were incubated at 25±1°C with 16/8 light/dark cycle at 15 µmol m^{-2} s^{-1} light density. Five culture vessels in each replication and four replications of each cultivar at each salinity level were employed. The experimental period was eight weeks, after that it was harvested and cleaned with tap water to remove agar remains and dried by tissue paper to remove the surface moisture.

Growth parameters

The growth parameters were noted on the shoot number, shoot length and fresh and dry weight (dried in the oven 70ºC to 24 h) of regenerated plantlets. The results obtained at each salinity level were evaluated with control (no NaCl in media). The reduction in growth traits was calculated by the following formula:

Reduction %= [(control- treat)/control] ×100

Estimation of chlorophyll content

Chlorophyll contents (Chlorophyll a, Chlorophyll b, Total chlorophyll) of the flag leaves were estimated according to Arnon (1949). Chlorophyll was extracted from 1 g fresh weight leaves with 80% aqueous acetone using mortar and pestle to grind the tissues. The suspension was decanted into centrifuge tubes and centrifuged at 4000 rpm for 3 min. The clear green solution was then filtrated from the colorless residue by Whatman filter paper (No. 2), and the volume made up to 20 ml with 80% acetone. The optical density (O.D) of this solution was determined against 80% acetone as blank using a spectrophotometer at 645 and 663 nm (Arnon, 1949). The chlorophyll a and b were determined according to the formula proposed by Arnon (1949) as follows:

Chlorophyll a µg/g = 12.71 (A_{663}) - 2.58 (A_{645}) × V/W

Chlorophyll b µg/g= 22.8 (A_{645}) - 4.67(A_{663}) × V/W

Total chlorophyll µg/g= 20.2 (A_{663}) + 8.02(A_{645}) × V/W

Where, V is the volume extract in ml and W is the fresh weight of sample in g

Statistic analysis

The factorial experiment (2×2×4) was designed in a completely randomized design (CRD) with four replicates. Data were analyzed by SAS program and the mean separation by the least significant difference test (LSD) at p=0.05 level.

RESULTS

Shoots number and Shoot length

The response of explants for shoots regeneration and shoot length under NaCl stress is shown in Figure 1. The number of shoots differed with the varied salinity level, when the NaCl level increased (75 mM), the average number of shoots was decreased (from 3.12 to 1.1) (Figure 2), and the reduction percentage decreased in treatments (25, 50 and 75 mM) respectively in 25, 47 and 65% in comparison with the control (Figure 3).

No differences were noticed between two cultivars in shoots number, where the number of shoots were 2.059 and 2.056 in Pearl and Beril (Table 1). The cotyledon explant produced higher shoots than the shoots raised from hypocotyls, where cotyledon and hypocotyl explants produced 2.32 and 1.78 shoots, respectively (Figure 4).

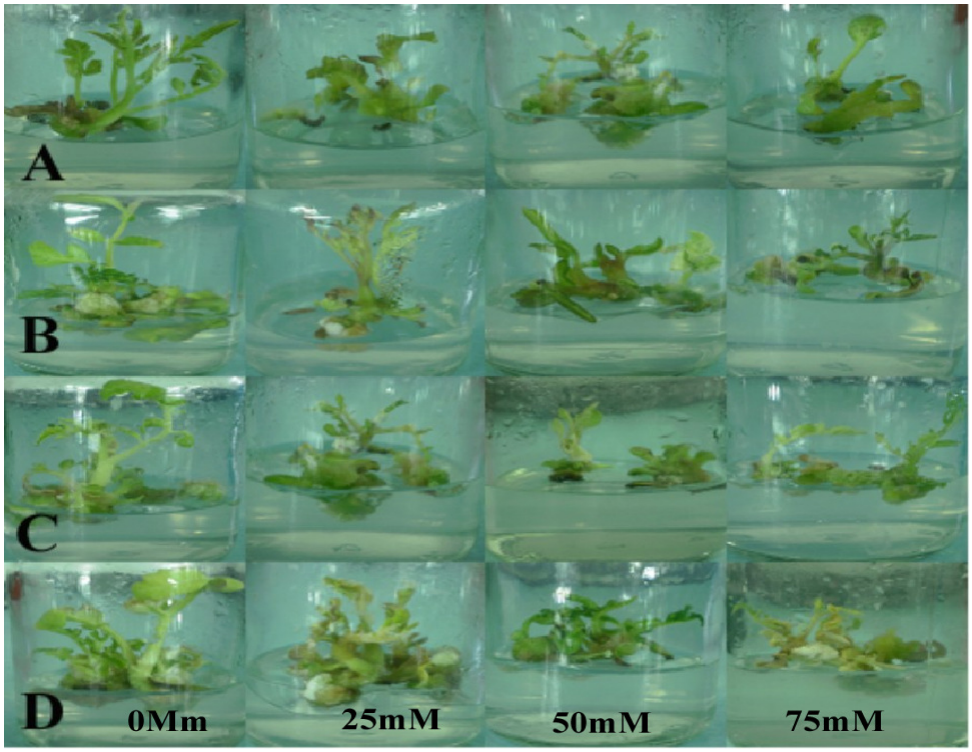

Figure 1. Growth and development of *in vitro* tomato explants under different levels of NaCl mM. (A-B) Hypocotyls and cotyledons of Pearl cultivar and (C-D) Hypocotyls and cotyledons of Beril cultivar.

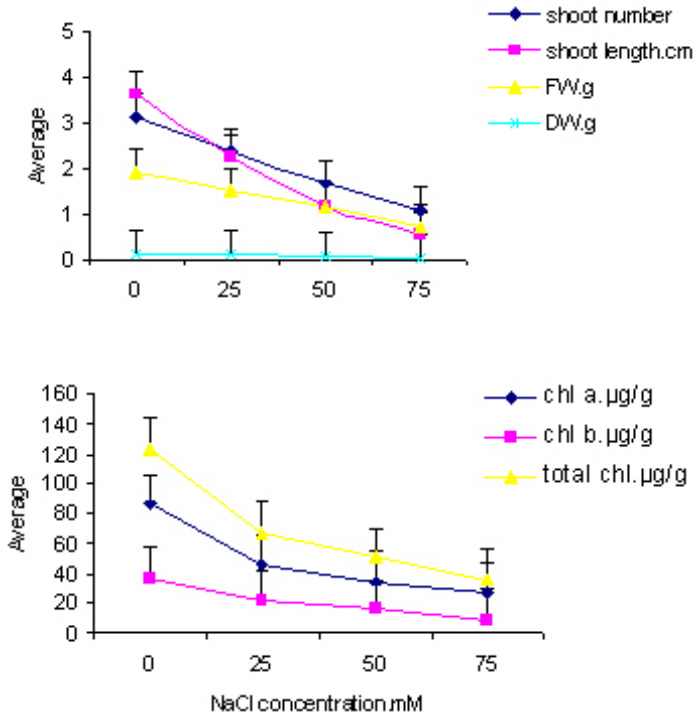

Figure 2. Effect of NaCl on growth traits in two tomato cultivars in vitro after eight weeks from incubation.

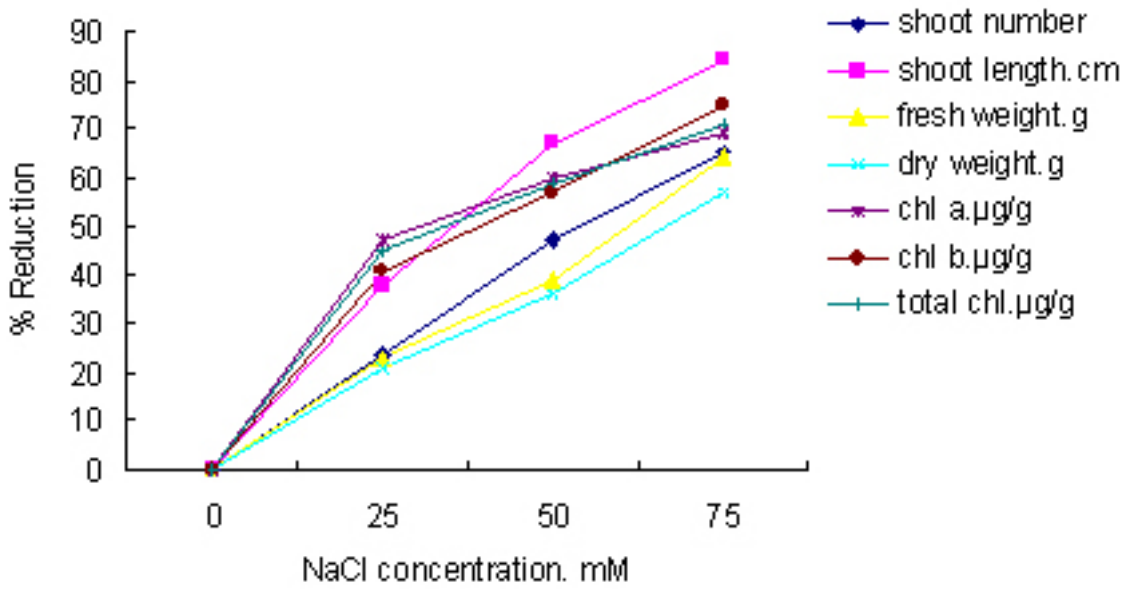

Figure 3. Reduction percentage in growth traits in two tomato cultivars under different level of NaCl (mM).

Table 1. Interaction between cultivars, explants and NaCl concentration (mM) on shoot number and shoot length traits *in vitro* after eight weeks of incubation (mean±SE).

NaCl (mM)	Cultivar			
	Pearl		Beril	
	Shoot number	Shoot length (cm)	Shoot number	Shoot length (cm)
Hypocotyls				
0	2.83±0.214	2.93±0.228	2.68±0.143	3.00±0.235
25	2.18±0.214	1.88±0.180	1.85±0.086	2.13±0.110
50	1.4±0.10	0.62±0.035	1.45±0.086	1.54±0.057
75	0.93±0.075	0.41±0.026	1.08±0.075	0.46±0.085
Cotyledons				
0	3.33±0.143	4.63±0.160	3.65±0.145	3.90±0.135
25	2.58±0.170	2.50±0.274	2.85±0.298	2.45±0.225
50	1.93±0.143	0.99±0.075	1.82±0.213	1.58±0.103
75	1.33±0.143	0.57±0.051	1.08±0.075	0.82±0.067
Mean	**2.059a**	**1.81b**	**2.056a**	**1.98a**

Shoots length gradually decreased with increased NaCl in growth medium (Table 1). The shoot length on media without NaCl (control) was noted in 3.61 cm whereas at high NaCl level (75 mM) it was 0.56 cm (Figure 2). The percentage reduction in shoots length decreased to 38, 67, and 85% in treatments 25, 50 and 75 mM respectively (Figure 3). Between the two cultivars, the shoot length in Beril was noted in 1.98 cm, which was significantly different with the Pearl (1.81 cm) (Table 1), and cotyledon explants were the best in shoot length than hypocotyl explants (Figure 4).

Fresh and dry weight

The shoots fresh weight was affected by increased NaCl

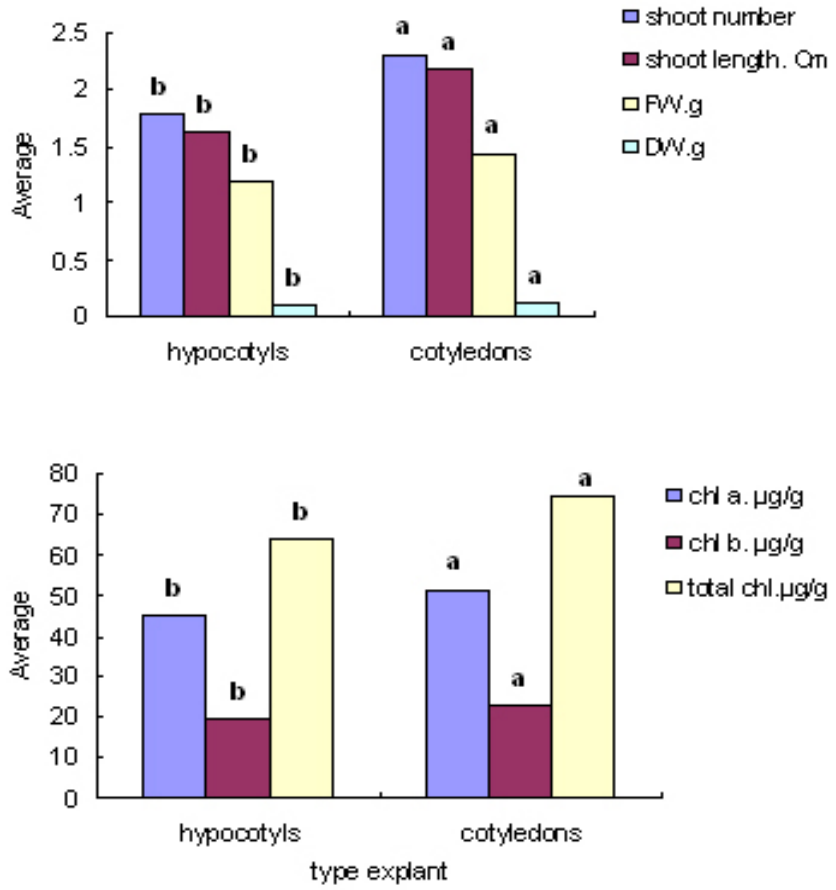

Figure 4. Explant performances on growth traits in two tomato cultivars under different level of NaCl (mM). In the same column mean value followed by the same letter are not significantly different at p=0.05 by LSD.

level in the growth medium (Table 2). Differences exist significantly between NaCl treatments shown in Figure 2 and the percentages of decline in fresh weight were recorded as 23, 39 and 64% on media having 25, 50 and 75 mM NaCl in comparison with the control (Figure 3). Similar pattern was shown in shoots dry weight under NaCl levels (Table 2), and the percentages of reduction in dry weight were 23, 36 and 56% on treatment 25, 50 and 75 mM NaCl respectively (Figure 3). The cultivars showed significant differences in fresh and dry weight, and in this regard Beril was found superior than Pearl (Table 2), and the explants also showed varied response to different NaCl supplemented media, where the cotyledon achieved the best values in fresh and dry weight compared with the hypocotyls (Figure 4).

Chlorophyll content

The chlorophyll contents of *in vitro* raised plantlets, was greatly influenced by NaCl treated media. Chlorophyll *a*, *b* and total chlorophyll content decreased with increased NaCl level in the medium (Table 3). The average chlorophyll *a* notably increased in control treatment (86.04 µg/g) while it decreased (26.83 µg/g) at high NaCl (75 mM) media (Figure 2), with reduction rate estimate of 69%, while in 25 and 50 mM was 47 and 60% respectively (Figure 3).

Chlorophyll *b* content was low compared with chlorophyll *a* at all treatments and the content of chlorophyll *b* also followed similar trend of chlorophyll *a*, where increase NaCl level led to decrease in chlorophyll *b* content. The reduction rate comparison with control was noted in 42, 58 and 76% in the treatments of 25, 50, and 75 mM respectively (Figure 3).

Between the two cultivars, Beril was found superior in chlorophyll content *a*, *b* and total chlorophyll, where Beril achieved the following rates of 52.6, 21.5 and 73.1 µg/g while Pearl achieved 44.81, 20.6 and 65.41 µg/g of chlorophyll *a*, *b* and total chlorophyll respectively (Table 3).

On the other hand, cotyledon explants showed

Table 2. Interaction between cultivars, explants and NaCl concentration (mM) on fresh and dry weight traits *in vitro* after eight weeks of incubation(mean±SE).

NaCl (mM)	Cultivar			
	Pearl		Beril	
	Fresh weight (g)	Dry weight (g)	Fresh weight (g)	Dry weight (g)
Hypocotyls				
0	1.54±0.092	0.12±0.005	2.00±0.107	0.15±0.012
25	1.18±0.037	0.10±0.0041	1.51±0.049	0.11±0.0041
50	1.05±0.015	0.09±0.003	1.17±0.039	0.09±0.007
75	0.45±0.013	0.06±0.0065	0.68±0.034	0.06±0.005
Cotyledons				
0	2.01±0.038	0.15±0.004	2.09±0.132	0.152±0.093
25	1.58±0.052	0.11±0.005	1.66±0.038	0.120±0.004
50	1.18±0.027	0.09±0.003	1.29±0.048	0.09±0.005
75	0.80±0.064	0.07±0.006	0.86±0.040	0.07±0.004
Mean	**1.22b**	**0.098b**	**1.41a**	**0.106a**

Table 3. Interaction between cultivars, explants and NaCl concentration (mM) on Chlorophyll content (*a*, *b* and total chl) *in vitro* after eight weeks of incubation (mean±SE).

NaCl (mM)	Cultivar					
	Pearl			Beril		
	Chl a (µg/g. fw)	Chl b (µg/g. fw)	Total chl. (µg/g. fw)	Chl a (µg/g. fw)	Chl b. (µg/g. fw)	Total chl (µg/g. fw)
Hypocotyls						
0	79.6±0.77	36.15±0.43	115.8±1.2	85±1.48	29.65±2.17	114.65±3.2
25	35.6±0.68	16.45±0.30	52.1±0.97	49.45±1.77	22.65±0.41	72.1±1.96
50	33.35±0.34	14.95±0.21	48.3±0.53	28.75±0.49	16.95±0.24	45.7±0.64
75	20.75±0.31	12.1±0.33	32.85±0.62	25.7±0.79	6.2±0.32	31.9±0.69
Cotyledons						
0	82.95±0.98	39.45±0.31	122.4±0.99	96.6±0.56	43.5±0.21	140.1±0.72
25	44.25±0.91	17.85±0.33	62.1±1.12	53.48±0.36	30.7±0.24	84.15±0.59
50	34.7±0.97	16±0.26	50.7±1.19	40.30±0.70	15.9±0.29	56.20±0.90
75	27.3±0.44	11.85±0.44	39.15±0.66	33.55±0.82	6.45±0.13	40±0.86
Mean	**44.81b**	**20.6b**	**65.41b**	**51.6a**	**21.5a**	**73.1a**

excellent chlorophyll content (*a*, *b* and total chlorophyll) than hypocotyls. The chlorophyll contents in the shoots produced from cotyledons explants (*a*, *b* and total chlorophyll) were noted to be 51.63 (chlorophyll a), 22.7 (chlorophyll b) and 74.35 µg/g (total chlorophyll) while in the shoots produced from hypocotyls, the contents of chlorophyll were 44.77, 19.38 and 64.16 µg/g of chlorophyll *a*, *b* and total chlorophyll respectively (Figure 4).

DISCUSSION

Plant tissue culture techniques is good tools for salinity studies, particularly for characterization and for obtaining salt tolerance plants (Cano et al., 1998). *In vitro* shoot morphogenesis to evaluate salt tolerance in cultivated tomato is a very important method to evaluation and screening for salt stress. Increased salinity in growth medium led to reduction in shoots number, shoots length,

fresh weight, dry weight and chlorophyll contents. These results agrees with the results reported by Mercado et al. (2000), where it was observed that presence of NaCl in the media strongly inhibited shoot regeneration, and Shibli et al. (2007) found that growth parameters were adversely affected by increased salinity in the medium in both Roma and Patio tomato cultivars. Similar results was reported by Hassan et al. (2008), where they noted decrease in fresh and dry weight of tomato and mung bean shoots strongly related with salt stress. El-Enany (1997) found that high level of salinity inhibited shoots regeneration from hypocotyls and cotyledons, and fresh and dry weight reduced with increased salinity in growth medium. Siler et al. (2007) studied the effects of salinity on growth and morphogenesis of *Centaurium erythraea in vitro*; they found that the high salt concentrations were effective in induction of auxiliary and adventitious buds on shoots. On the other hand, Vijayan et al. (2003) reported that increase in salinity level inhibited the growth and development of mulberry shoots *in vitro*. Similar results were reported by Potluri and Prasad (1994) in potato, Kashyap and Sharma (2006) in *Morus alba* and Erturk et al. (2007) in cherry, where they found degradation in shoot length, shoots number and dry matter with increased salinity in growth medium. The reduction of plant growth *in vitro* under salt stress may be attributed to decline ability of explants to take up the water (Chapin, 1991) and nutrition elements imbalance (Munns and Termaat, 1986; Marschner, 1995), addition to accumulation of toxic ions in plant tissues (Marschner, 1995). Moreover, high level of salinity causes hyperosmotic stress and ion disequilibrium in cell producing secondary effects like reduction of turgor below the yield threshold of cell wall resulting in growth cessation (Yokoi et al., 2002).

Chlorophyll content in the plant play a major role in increasing photosynthesis efficiency and improve plant growth. Chlorophyll *a* become the major pigments in plant, and plays a major role in photosynthesis; absorbing and reacting with the visible light (Bower and Leegood, 1997). Change in chlorophyll content in plants under the effect of salinity is the most important features of biochemical response (Rao et al., 1991). The results indicated that chlorophyll content declined in both tomato cultivars under the salinity stress and chlorophyll *b* seems to be more affected than chlorophyll *a*. The decreases in chlorophyll content noticed in salt-stressed plants may be due to a decrease in chlorophyll synthesis or to an increase in chlorophyll degradation (Santos, 2004), whereas the membranes and its stability seldom remains right under salt stress conditions (Ashraf and Foolad, 2005). The result also agrees with what was found by El-Meleigy et al. (2004) and Amini and Ehsanpour (2006) in tomato. Siler et al. (2007) studied the effects of salinity on chlorophyll content in (*Centaurium erythraea* Rafn.), where they found that high salt concentrations were effective in chlorophyll *a* and *b* contents and total chlorophyll decreasing trend with increasing supply of NaCl in the growth medium. A similar result was reported in cherry plant by (Erturk et al., 2007) and *Catharanthus roseus* by (Abdul Jaleel et al., 2008). The results also agrees with those reported by (Molazem et al., 2010; Iqbal et al., 2006; Ashraf and Foolad, 2005), who reported that chlorophyll content was decreased under saline conditions.

Conclusion

Salinity tolerance of tomato cultivars can be successfully evaluated *in vitro* as a promising substitute for conventional field evaluations. The results demonstrated that NaCl stress greatly influenced the *in vitro* performances of two tomato cultivars and the explants. Response of different parameters gave the varied results with the application of NaCl treatments in the media. The impact noted in Beril showed the best responsive cultivar than Pearl whereas cotyledon regarded the suitable explants than hypocotyls at all parameters tested. The findings may contribute to better multiplication from cotyledon explants in Beril cultivar to raise a salt tolerant line for future use. This study needs further refinement to find out more insight on the Beril cultivars and their *in vitro* sustainability, and if possible check for subsequent field performances where salinity level is a major obstacle to tomato production.

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