

A rapid screening method for salt stress tolerance of onion genotypes

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Abstract

Salinity is one of the major limitations for onion growth and productivity all over the world. There are several methods to determine salt stress tolerance of plants such as germination tests, pots trials, *in vitro* experiments *etc.* This study was conducted for optimisation of rapid and practical method to compare onion genotypes under salinity conditions. In the first stage of the study, three explant types and twenty one combinations of plant growth regulators were tested for optimization of callus protocol. Callus induction percentage (%), callus fresh weight (mg) and callus dry weight (mg) were measured on the 31st day. According to results, the best medium (MS + 2 mg/L 2,4-D + 0,5 mg/L BAP) and explant type (mature zygotic embryo) for the proliferation of callus were determined. After choosing the best hormone and explant type, the effect of salinity on callus induction was tested using different level of NaCl. 'Akgun-12' consistently performed the best in callus culture. Responses of onion cultivars were different for different parameters. The proposed method was simple to perform, as no long time is required, and offers a possibility to screen genotypes in any time of year.

Key words: Onion, *Allium cepa* L., *in vitro*, callus, salinity, embryo, explant

Introduction

Onion (*Allium cepa* L.) is an important crop that is now cultivated globally. According to the most recent data of the United Nations Food and Agriculture Organization (FAO), worldwide onion production was approximately 85.7 million tons in 2013 from 4.43 million hectares. According to the FAO, Turkey produces 1.9 million tons of onions annually, which is 2.2% of the world onion production, and it ranks as the 6th largest onion producer (FAO, 2013). Many important crops are glycophytes that show susceptibility to soil salinity. Seed germination and emergence are critical to the survival of plants in salt-affected areas (Khan, 2002). Among crop species, different threshold tolerances (electrical conductivity, EC) and reduction rates of yield are recorded and this indicates that there is a variation in salt tolerance mechanisms (Chinnusamy and Zhu, 2005). According to Maas and Hoffman (1977), onion is very sensitive to EC values as low as 1.2 dSm⁻¹.

There are different methods to determine salt stress tolerance of plants. Screening of salt tolerance levels of plants is difficult in the field conditions because of lateral, vertical and temporal variability in salt distribution within the soil profile. In addition, plant salt tolerance varies with ontogeny, the growth parameter measured and environmental factors (Bekheet *et al.*, 2006). Pot trials are very common method for salt stress tolerance, but, this method may be very costly in some circumstances. However, tissue culture techniques can be used for this aim with other purposes such as genetic transformation (Lee *et al.*, 2015), *in vitro* selection (Tripathi *et al.*, 2008), plant regeneration (Mendi *et al.*, 2009), double-haploid plant production (Tantasawat *et al.*, 2015), *etc.* Plant response to abiotic stress is a complex phenomenon, which could be approached efficiently through *in vitro* culture. Plant tissue culture techniques provide a promising and feasible approach to develop salt tolerant plants (Benderradji *et al.*, 2012). *In vitro* screening or selection studies for salt tolerance in onion are very limited (Bekheet *et al.*, 2006).

Optimization of a rapid, time-dependent and low-cost method for screening of onion genotypes under salinity conditions was the objective of this study. The study was planned in two stages: (1) Choosing of suitable explants type and combination of plant growth regulators; (2) Comparison of callus induction performances of onion genotypes in *in vitro* salinity conditions.

Material and method

Preparation of explants: The green shoots (SE), root pieces (RE) and mature embryos (MEE) of 'Akgun-12' cultivar were used as explant resources (Fig.1). Onion seeds were surface sterilized by (a) holding them under running water 5 minutes, (b) immersing in sterile water containing three drops of Tween-20 per 100 mL and 30% commercial bleach (<4% hydrochloric acid) for 15-20 minutes with continuous agitation followed by (c) 5-10 washings with sterile tap water. Then, they were surface disinfested for second time by (d) 70% alcohol for 2 minutes, then rinsed (e) in sterile deionized water 4-5 times. Seeds were cultured on simple MS (Murashige and Skoog, 1962) medium up to seedling stage. The media contained 30 g/L sucrose and 7 g/L agar as a solidifying agent. pH adjusted at 5.7. The cultures were kept at 25±2 °C under cool white fluorescent light with a 16 h photoperiod. After one month of seedling development, *in vitro* grown sterile seedlings were used as the shoot and root explant source. For obtaining another explant type, after disinfection, seeds were stored in the sterile water at 4°C for 5-6 hours. The embryos were aseptically excised using a binocular. The part of the embryo containing the radicle, the shoot apex and the lower part of the cotyledon (Van der Valk *et al.*, 1992) was separated from the main part of the cotyledon.

Preparation of plant growth regulators combinations: MS basal media was supplemented with different concentration of 2,4-D (2,4-Dichlorophenoxyacetic acid), Naphthalene acetic acid (NAA), 6-Benzylaminopurine (BAP), and Kinetin (K). A total of

21 treatments, MS01 to MS21, were tested for each explant type (Table 1). All the media contained 30 g/L sucrose and 7 g/L agar as a solidifying agent. pH adjusted at 5.7. The cultures were kept at 25±2 °C in the dark.

Table 1. The combinations of auxin and cytokinin (mg/L)

Media	Auxin		Cytokinin	
	2,4-D	NAA	BAP	K
MS-1	0	0	0	0
MS-2	1	0	0	0
MS-3	1	0	0.5	0
MS-4	1	0	0	0.5
MS-5	2	0	0	0
MS-6	2	0	0.5	0
MS-7	2	0	0	0.5
MS-8	1	0	1	0
MS-9	1	0	0	1
MS-10	2	0	1	0
MS-11	2	0	0	1
MS-12	0	1	0	0
MS-13	0	1	0.5	0
MS-14	0	1	0	0.5
MS-15	0	2	0	0
MS-16	0	2	0.5	0
MS-17	0	2	0	0.5
MS-18	0	1	1	0
MS-19	0	1	0	1
MS-20	0	2	1	0
MS-21	0	2	0	1

The plant growth regulators and explant types had 21 and 3 levels, respectively. Five explants of each type were cultured per dish for callus induction. At least 6 dishes were used per treatment. Data were collected on 31st day of culture. For determination of callus inductions differences among the medias and explants, callus induction percentage (CIP), callus fresh weight (CFW) and callus dry weight (CDW) were used as the parameters. The callus induction percentage (CIP) was calculated as follows:

$CIP (\%) = \text{Number of Explants with Callus} / \text{Total Number of Explants}$

Comparison of callus induction performances: For the screening study, the callus induction of several onion cultivars was tested at different NaCl concentrations using best explant type and supplemented media. The seeds of ‘Kantartopu-3’ (Knt.), ‘Akgün-12’ (Akg.), Besirli-77 (Bsr.) and ‘İmrli Kırmasi-15’ (Imr.) cultivar were obtained from the Atatürk Central Horticultural Research Institute in Yalova. ‘Metan-88’ (Mtn.) and ‘Beyaz Bilek’ (Bbl.) seeds were provided by the MTN Seed Company in Balıkesir, Turkey. ‘Texas Early Grano’ seeds were provided by the commercial seeds sales office. For comparison of callus induction performances, NaCl was added to the best medium which was determined at end of the experiment-1. Salinity levels of the mediums were adjusted to 0, 1.2, 2.4, 4.8 and 9.6 dSm⁻¹ by using different NaCl concentrations. Cultivars and salinity had 7 and 5 levels, respectively. Five mature embryo explants which was determined at the experiment-1, were cultured per dish for callus induction. At least 6 dishes were used for per treatment. Data were collected on 31st day of culture. For determination of callus inductions differences among

the cultivars, CIP, CFW and CDW were used as the parameters.

The experiment was conducted in the laboratory of the Department of Vegetable Breeding and Tissue Culture at the Atatürk Central Horticultural Research Institute. For comparison of multiple means, one-way ANOVA and the LSMeans Differences Student’s t test were used. Significant difference of statistical tests was tested at $P < 0.01$.

Results and discussion

Mature embryo explants showed the earliest signs of callus formation after six days of culture, but shoot and root started to initiate after two weeks of culture. Callus type was scored as no callus, compact (white and nodular type) and friable. The compact callus were observed at extremely low rates (from only three explant). These calluses were not taken into consideration.

The results of variance analyses for the measured characters are presented in Table 2 and 3. The callus induction percentage, callus fresh weight and callus dry weight were significant for three factors (media, explant types and their interaction), so while interpreting the results, averages of main treatments (media and explant) are not labeled with letters.

Twenty one media comprising of MS basal salts along with different auxin and cytokinin concentrations were used to test their efficiency for callus culture with three types of explant. Out of these media, media MS-6 (containing 2 mg/L 2,4-D and 0.5 mg/L BAP) was found to be the best with mature embryo explant on callus induction efficiency of 70.67% (Table 4). The callus inductions were observed in the eighteen different media when mature embryos were used. Whereas, callus inductions were observed only in two media (MS-6 and MS-11) when root explants used. MS-5 and MS-6 media had best results for shoot explant. The highest mean callus induction percentage (21.40%) was obtained from the mature embryo explant, while the lowest callus induction percentage (0.57%) was obtained from the root explant.

Callus weight significantly changed depending on the interaction of media and explant types. For evaluation of callus fresh and dry weight, two “mean value” were calculated (Table 4). The mean-1 value represents average of all media (twenty-one). The mean-2 value represents average of media on which callus was induced. The highest callus weight (220.33 mg/per explant) was obtained from the treatment of MS-6 (containing 2 mg/L 2,4-D and 0.5 mg/L BAP using mature embryo explant). The highest mean-1 and mean-2 callus fresh weight was observed from mature embryo explant with 135.36 and 149.52 mg/per explant, respectively, whereas the lowest was observed from root explant (2.15 and 35 mg). In general, the mature embryos produced more heavy callus than the others. Also, preparing of shoot and root explant was a process that required certain time period because

Table 2. Two-way analysis of variance for experiment 1

Source	F Ratios			
	DF	CIP	CFW	CDW
Media	20	25.2277*	102.1781*	55.8215*
Explants	2	198.2124*	1889.550*	888.3968*
Media x Explants	40	10.6088*	57.8469*	30.7474*
Error	126			

*Significant ($P < 0.01$) F: Freedom, DF: Degree of freedom, CIP: Callus induction percentage, CFW: Callus fresh weight for each callus CDW: Callus dry weight for each callus.

Table 3. Two-way analysis of variance for experiment 2

Source	F Ratios			
	DF	CIP	CFW	CDW
Cultivar	6	2503.5*	1 229.9*	2 735.5*
Salinity	4	35036.1*	27 992*	43 534.8*
Cultivar x Salinity	24	453.8*	479.4*	511.1*

*Significant ($P < 0.01$) F: Freedom, DF: Degree of freedom, CIP: Callus induction percentage, CFW: Callus fresh weight for each callus, CDW: Callus dry weight for each callus.

of aseptic seedlings. However, in mature embryo explant process, explants can be prepared in one or two hours.

It was reported that 1.0 mg/L 2,4-D concentration is optimal concentration for callus formation in *Allium* species (Luciani *et al.*, 2006). According to our results, mature embryo explant and MS-6 media (MS+2 mg/L 2,4D + 1 mg/L BAP) were most suitable for the callus induction and callus weight as compared to other two explant types and twenty combinations of plant growth regulator in order to obtain high rate of callus. Thus one should determine the best medium for callus induction by considering both the explant type and medium together.

Callus induction in monocots requires long time for its initiation (Geier, 1986), whereas in this study, callus formation from mature embryo explants could be observed after six days of culture. These data were similar to the results reported by Zheng *et al.* (1998). It was reported that the induction of callus was difficult and the

proliferation of initiated callus was very slow and somehow difficult to maintain in *Allium* species (Zheng *et al.*, 1998; Luciani *et al.*, 2006)

For determination of tolerance differences between cultivars using callus parameters, salt tolerance indexes were calculated based on CIP, CFW and CDW. Generally, all parameters were reduced by increased salt concentration (Table 5). That effect was more pronounced in 'Beyaz Bilek' and 'Kantartopu-3' than in other cultivars. For example, in the 2.4 dSm⁻¹ salt treatment, tolerance index of callus induction percentage (CIP_{TI}, %) of 'Beyaz Bilek' was only 16.65% after 31-days incubation. In all cultivars, maximum CIP_{TI} % was in the non-saline control. When explants were incubated with 4.8 and 9.6 dSm⁻¹ NaCl, callus percentage was more adversely affected than other treatments. 'Akgün-12' cultivar was more tolerant to salinity than other cultivars based on the results of all tolerance indexes. This result is in agreement with our previous report (Hanci *et al.*, 2012). In that study, seeds of same cultivars had been compared for germination period under the salinity conditions obtained using NaCl and CaCl₂. According to the results, 'Akgün-12' was more tolerant than other cultivars. Also, 'Beyaz Bilek' had been found more sensitive than the others to salt stress.

In summary, we conclude that the selection of explants and appropriate media improved callus induction. In the present study, the mature embryo explants of *A. cepa* responded well when

Table 4. Effects of plant growth regulators and explant types on callus induction, fresh weight and dry weight

Media	CIP (%)				CFW (mg)				CDW (mg)			
	(Explant)				(Explant)				(Explant)			
	SE	RE	MEE	Avr.	SE	RE	MEE	Avr.	SE	RE	MEE	Avr.
MS-1	-	-	-	-	-	-	-	-	-	-	-	-
MS-2	21.00 e	-	11.11g	11.04	82.00 kl	-	101.00 jk	61.00	18.00 l	-	26.00 k	14.67
MS-3	21.00 ef	-	12.44 fg	11.15	175.00 cd	-	174.00 cd	116.44	42.00 efgh	-	41.00 fgh	27.67
MS-4	22.33 e	-	11.11 g	11.15	154.33 efg	-	152.00 efg	102.11	39.00 gh	-	36.00 hi	25.00
MS-5	34.00 d	-	33.33 d	22.44	203.00 ab	-	208.00 a	137.00	54.00 abc	-	55.00 ab	36.33
MS-6	44.00 c	6 gh	70.67 a*	40.22	202.00 ab	35.00 m	220.33 a*	148.55	56.00 ab	4.67 m	60 a*	40.22
MS-7	33.00 d	-	44.44 c	25.81	133.00 hi	-	139.00 gh	89.56	30.00 ijk	-	35 hij	21.44
MS-8	-	-	11.11 g	3.70	-	-	144.00 fgh	48.00	-	-	35 hij	11.67
MS-9	-	-	10.77 g	3.59	-	-	114.00 ij	38.00	-	-	28 jk	9.33
MS-10	-	-	33.33 d	11.11	-	-	201.00 ab	67.00	-	-	54 abc	18.00
MS-11	-	6 gh	55.56 b	20.52	-	33.00 m	208.00 a	76.66	-	4.00 m	51 bcd	18.33
MS-12	-	-	-	-	-	-	-	-	-	-	-	-
MS-13	-	-	11.11 g	3.70	-	-	188.00 bc	62.66	-	-	47 cdef	15.67
MS-14	-	-	22.22 e	7.41	-	-	103.00 j	35.11	-	-	18 l	6.00
MS-15	11.11 g	-	22.22 e	11.11	110.00	-	105.00 ij	71.67	28.00 jk	-	18 l	15.33
MS-16	10.77 g	-	11.11 g	7.29	165.00 de	-	162.00 def	109.00	49.00 bcde	-	46 defg	31.56
MS-17	11.41g	-	33.33 d	14.91	109.00 l	-	113.00 ij	61.89	12 l.00	-	26 k	12.67
MS-18	-	-	11.11 g	3.70	-	-	169.00 cde	56.63	-	-	41.33 fgh	13.78
MS-19	-	-	-	-	-	-	-	-	-	-	-	-
MS-20	-	-	22.22 e	7.41	-	-	170.00 cde	56.67	-	-	45.66 defg	15.33
MS-21	-	-	21.15 e	7.38	-	-	169.67 cde	56.22	-	-	44.67 defg	14.89
Avr. ¹	9.98	0.57	21.40		61.60	2.15	135.36		15.59	0.41	33.70	
Avr. ²	-	-	-		139.49	34.00	149.52		36.37	4.34	37.25	
LSD	19.688				38.963				7.171			

*Means within a group (CIP, CFW, CDW) that have a different small letter are significantly different from each other. $P < 0.01$. CIP: Callus induction percentage, CFW: Callus fresh weight for each callus CDW: Callus dry weight for each callus, SE: Shoot explant, RE: Root explant, MEE: Mature embryo explant Avr.¹: represents results of all treatments. Avr.²: represents results of callus indicated treatments. LSD: Least Significant Difference

Table 5. Effect of plant growth regulators and explants types on callus induction, fresh weight and dry weight.

EC _e (ds/m)	CIP _{TI%}							
	Akg.	Knt.	Imr.	Bsr.	TEG	Mtn.	BBl.	Avr. ²
0.0	100 a	100 a	100 a	100 a	100 a	100 a	100 a	100
1.2	82.91b	49.99f	59.94d	83.32 b	83.28b	59.94d	49.97e	67.26
2.4	82.91b	16.66j	39.96g	66.65c	49.97f	19.98i	16.65j	41.91
4.8	49.75f	0 k	0 k	33.32h	0 k	0 k	0 k	11.87
9.6	16.58j	0 k	0 k	0 k	0 k	0 k	0 k	2.37
Avr. ¹ .	66.44	33.34	38.98	58.68	46.60	36.00	33.73	

EC _e (ds/m)	CFW _{TI%}							
	Akg.	Knt.	Imr.	Bsr.	TEG	Mtn.	BBl.	Avr. ²
0.0	100 a	100 a	100 a	100 a	100 a	100 a	100 a	100
1.2	72.72c	60.86e	57.14f	68.18d	52.17i	52.17i	80.00b	63.37
2.4	54.54h	47.82k	52.38i	54.54gh	43.47m	56.52fg	70.00d	54.14
4.8	50.00j	0 n	0 n	54.54gh	0 n	0 n	0 n	15.00
9.6	45.45l	0 n	0 n	0 n	0 n	0 n	0 n	6.50
Avr. ¹ .	64.54	41.73	41.80	55.73	39.00	41.81	50.00	

EC _e (ds/m)	CDW _{TI%}							
	Akg.	Knt.	Imr.	Bsr.	TEG	Mtn.	BBl.	Avr. ²
0	100 a	100 a	100 a	100 a	100 a	100 a	100 a	100
1	75.00b	33.33g	50.00	44.44d	44.44d	30.00h	42.85e	45.57
2	50.00c	22.22i	43.75c	44.44d	33.33g	30.00h	42.85e	37.97
5	50.00c	0 j	0 j	44.44d	0 j	0 j	0 j	13.57
10	37.50f	0 j	0 j	0 j	0 j	0 j	0 j	5.43
Avr. ¹ .	62.60	30.87	38.80	46.60	35.53	32.00	37.16	

*Means within a group (CIP_{TI%}, CFW_{TI%}, CDW_{TI%}) that have a different small letter are significantly different from each other. $P < 0.01$. CIP_{TI%}: Tolerance index for Callus induction percentage, CFW_{TI%}: Tolerance index for Callus fresh weight for each callus, CDW_{TI%}: Tolerance index for Callus dry weight for each callus. Knt='Kantartopu-3', Akg='Akgün-12', Imr.=Imralı Kırması, Bsr=Besirli-77, Mtn= Metan-88, BBl=Beyaz Bilek, TEG=Texas Early Grano. Avr.¹:represents results of all treatments. Avr.²:represents results of callus indicated treatments.

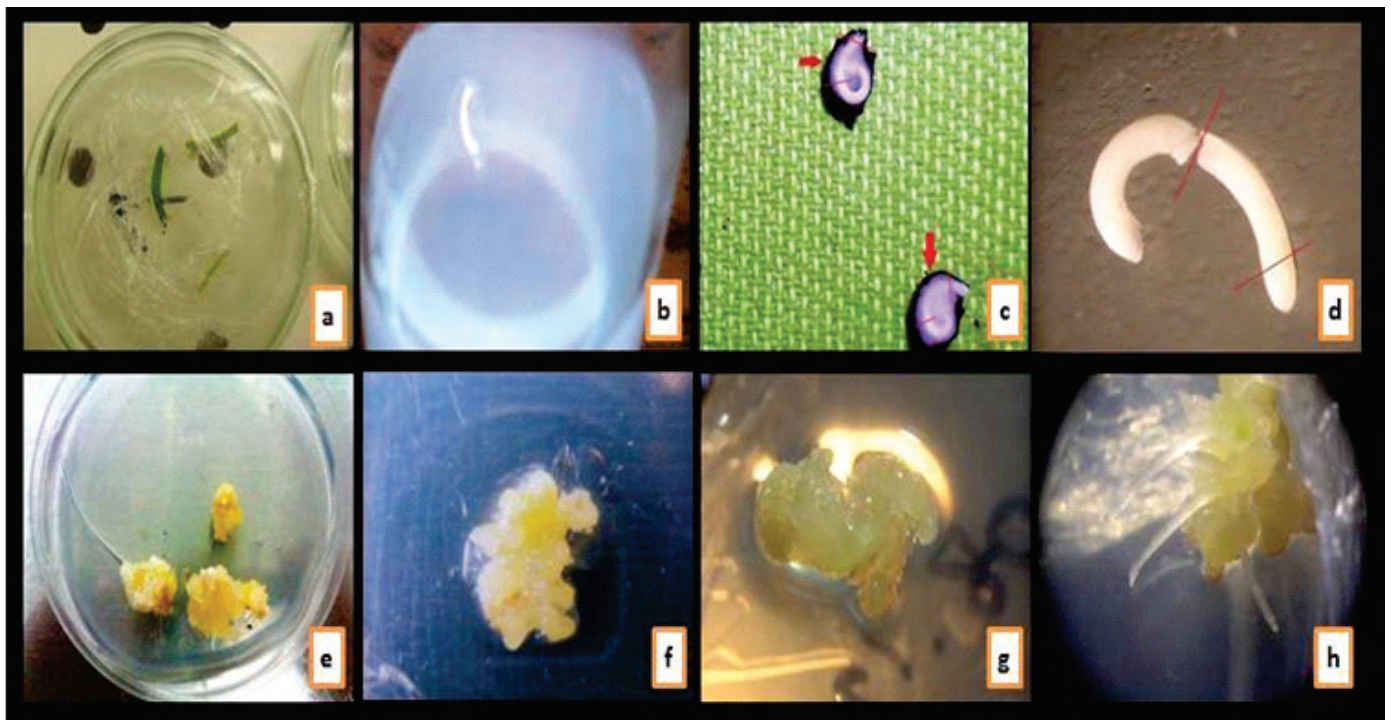


Fig. 1. (a) Shoot explant, 5-7 mm, (b) Root explant, 4-6 mm, (c) and (d) Mature embryo explants (between red lines) (e) Compact callus, (f) and (g) Friable callus from mature embryo after 20 days of culture (h) Callus induction from mature embryo after four weeks of culture

cultured on the MS medium supplemented with combinations of auxin (2,4-D) and cytokinin (BAP). 'Akgun-12' consistently performed the best in callus culture. This method can be used at any time of the year and results can be obtained at the end of first month. So, this method could be advised as rapid screening method for the salt stress studies in onion.

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