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Effects of saline and mannitol induced stress on some biochemical and physiological parameters of *Carthamus tinctorius* L. varieties callus cultures

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Abstract

Worldwide agricultural productivity is subjected to increase in environmental constraints, particularly drought and salinity. The aim of this study was to evaluate the physiological responses of safflower (*Carthamus tinctorius* L.) calli exposed to water stress and salinity in order to elucidate some acclimatory mechanisms. Therefore, different calli of safflower genotypes, G_1 (LRV-51-51), G_2 (Lesaf), G_3 (Gila), G_4 (Kino-76) and G_5 (Isfahan), were exposed to different concentrations of mannitol and NaCl for one month. The ongoing research was conducted in order to evaluate relative growth rate, relative water content, tolerances index, ion (Na^+ and K^+) and proline content and cell viability of genotypes. The results indicated that a significant decrease in callus growth, water content and cell viability occurred under both stresses with the highest reduction under mannitol-induced osmotic stress. Although the leached and retained Na^+ ion contents increased, the retained K^+ content decreased with increasing levels of saline. While under mannitol-induced osmotic stress, Na^+ remained unchanged but K^+ concentration decreased significantly. So, overall results indicated that the accumulation of Na^+ ions and osmolytes could play an important role in osmotic adjustment in safflower cells under saline stress. Also, among the studied genotypes, Gila genotype (G_3) and Kino-76 (G_4) showed higher cell viability, higher K^+, Na^+ and proline concentration. Furtheremore, both genotypes appeared to have a good efficiency in water retainment which can be considered as resistant cultivars to saline and mannitol iso-osmotic stresses respectively.

Keywords: Cell viability, Ion uptake, Mannitol, NaCl treatment, Safflower calli.
Abbreviations*:* 2,4-D_2,4-Dichlorophenoxyacetic acid; NAA_α-Naphthaleneacetic acid; BAP_6-Benzylaminopurine; MS_ Murashige and Skoog medium; RWC_Relative water content; RGR_Relative growth rate; TTC_2,3,5-Triphenyltetrazolium chloride.

Introduction

Safflower (*Carthamus tinctorius* L.) is a tap-rooted multipurpose crop which can tolerate environmental stresses including salinity and water stress (Lovelli et al., 2007). It is one of the most important oil seed cultivated plant used for edible oil production in the world (Dwivedi et al., 2005). The importance of oil crops such as safflower has increased in recent years, especially with the interest in the vegetable oil for the human consumption (Dordas and Sioulas, 2008). Generally, safflower is cultivated on marginal lands that are relatively dry and deprived in order to benefit fertilizer inputs and irrigation. Also, attempts to improve seed yield and quality by developing new genotypes and agronomic practices are underway throughout the world (Dordas and Sioulas, 2008). Plants exposed to stresses may undergo changes in their metabolism in order to be adjusted according to their prevailing environmental conditions, an important skill for a sedentary organism. Agricultural productivity worldwide is subjected to increasing environmental constraints, particularly to drought and salinity due to their high magnitude of impact and wide distribution (Kaviani, 2008; Karimi et al., 2011). Salt and drought stress leads to the suppression of plant growth and development, membrane leakage, ion imbalance or disequilibrium, enhanced lipid per oxidation and increased production of reactive oxygen species which are scavenged by both enzymatic and non-enzymatic reactions (Summart et al., 2010; Zebarjadi et al., 2010). Furthermore, drought and salinity stress are by far the most important environmental cues in agriculture and many efforts have been made to improve crop productivity under water-limiting and saline conditions (Cattivelli et al., 2008; Karimi et al., 2011). Drought and saline induced osmotic stress, triggers a wide range of perturbations ranging from growth and development disruption to the modification of ion transport and uptake systems (Lutts et al., 1996; Bajji et al., 2000; Zebarjadi et al., 2010; Karimi et al., 2011).In order to maintain homeostasis during stress condition, plants exhibit physiological, biochemical and molecular responses at both the cellular and whole plant levels (Shao et al., 2007; Dufty et al., 2002; Ghasempour et al., 1998; Ghasempour et al., 2007). Generally, the plants accumulate some kind of organic and inorganic solutes in the cytosol to raise osmotic pressure and thereby maintain both turgor and the driving gradient for water uptake (Patade et al., 2008; Ghasempour and Kianian, 2007). These compounds, which include proline, serve as osmoprotectants under stress conditions, maintain membrane structure and act as free radical scavengers preventing lipid

peroxidation or as regulators of K+ channels in stomata (Hasegawa et al., 2000; Parida and Das, 2005; Ghasempour and Kianian, 2007). Also, the tolerance ability of some plants like rice (*Oryza sativa* L.) to salt or drought stress depends on plant genotypes. For example, rice varieties (Pokkali, Indica and Nonabokra) having high endogenous ABA levels during stress conditions, are classified as highly salt-tolerant ecotypes, while the majority of high yielding cultivars such as M-1-48, IR-29, IR–36 and IR72 are salt-sensitive (Moons et al.,1995; Summart et al., 2010). In vitro culture technique serves as a useful tool to study the biochemical and physiological response of undifferentiated callus to salinity and drought stress at the cellular level (Bajji et al., 1998; Elkahoui et al., 2005; Ghasempour et al., 2007). The plant cell culture studies also allow isolation and selection of salt and drought tolerant lines to elucidate mechanism of tolerance operating at cellular level (Bajji et al., 1998; Elkahoui et al., 2005; Niknam et al., 2006; Errabi et al., 2007) and the possibility of developing salt and drought tolerant lines. Moreover, the distinction between the ionic and the osmotic component of salt stress by the addition of ionic or non-ionic (such as polyethylene glycol (PEG)) solutes to the culture media, and the relationship between surviving abilities of cultured cell lines and their growth properties can be served by in vitro culture techniques. Undifferentiated cells and callus cultures eliminate complications associated with genetic and morphological variability inherent to different tissues in whole plants (Parida and Das, 2005; Shao et al., 2007; Ghasempour et al., 2007). Understanding of plant ability in fight to stresses open a way for crops manipulations for their ability in tolerance, adaptation or resistance to stresses (Lutts et al., 2004; Parida and Das, 2005; Movahhedy-Dehnavy et al., 2009). The objective of the present study was to gain information on the comparative effects of saline and water stress on cell viability, cell growth and cellular recovering abilities using callus obtained from five genotypes of safflower exhibiting contrasting levels of salinity resistance. These parameters are analyzed in relation to osmotic adjustment and ion accumulation in stressed tissues.

Results

Analysis of variance (ANOVA) for all measured traits of salt and drought stressed genotypes revealed significant differences between the five genotypes. Furthermore, the genotype × medium interaction also showed a significant difference for all the measured factors on each stressed traits (Table 1). The results indicated the presence of a considerable amount of genetic variation among the studied genotypes, especially under drought stress conditions.

Callus growth and water content

Relative growth rate (RGR) appeared to be remarkably influenced by genotype, since a significant difference among genotypes was recorded even in the absence of stress (Fig. 1). Calli obtained from G_3 and G_5 exhibited the highest and lowest RGR values, in spite of what the NaCl dose was. In the presence of the mannitol, G_3 exhibited the highest RGR values, while G_2 exhibited the lowest value of mannitol-induced osmotic stress (Table 2). NaCl- and mannitol-induced stress decreased RGR among all the genotypes. Mannitol-induced osmotic stress seemed to be more harmful to G_1, G_2 and G_4 callus RGR than NaCl-induced stress (Table 2, Fig. 1). In contrast, callus RGR of G_3 and G_5 decreased more under salt-induced stress than mannitol-induced osmotic stress. RGR reduction corresponded to 75, 97, 84, 70 and 89% of the control at the 200 mM concentration of NaCl and to 89, 101, 81, 82 and 83% of the control in the presence of mannitol iso-osmotic concentration in G_1, G_2, G_3, G_4 and G_5 respectively (Table1, Fig. 1).

The decrease of callus water content was observed under both NaCl- and mannitol-induced stress. The highest reduction was noticed under mannitol-induced osmotic stress at all the experimented doses and it reached to about 79, 75, 73, 75 and 80% of the control in the presence of the highest mannitol concentration (505 mM) while it to about 75, 95, 64, 85 and 63% of the control in the presence of NaCl iso-osmotic concentration (300 mM) in G_1, G_2, G_3, G_4 and G_5 respectively (Fig. 2). Callus growth was also expressed as a tolerance index (TI) to eliminate inherent differences associated with the RWC of the five genotypes in response to implemented stress. Increasing mannitol and NaCl concentration was associated with a reduction in TI for safflower callus (Fig. 3). In mediums with NaCl, G_4 and G_2 calli always exhibited the highest and lowest TI values respectively. Whereas, in the presence of mannitol, the highest and lowest TI values were observed in G_3 and G_2, respectively (Fig. 3).

Sodium and potassium concentration

As shown in Table 2 and figure 5, the K^+ contents declined significantly ($p<0.05$) in cotyledon derived calli under NaCl and mannitol induced osmotic stress. In contrast, Na^+ content was increased significantly as NaCl concentration augmented (Fig 4). In the absence of stress, Na^+ concentration differed significantly ($P < 0.001$) among the genotypes, and was lower in G_4 than in other genotypes. A highly significant difference was recorded among the effects of mannitol and NaCl in relation to the callus ion concentration. The exposure to NaCl induced an increase in Na^+ (Fig. 4) as well as a decrease in K^+ concentrations (Fig. 5). Na^+ concentration increased to about 6.03, 4.30, 4.10, 7.65 and 5.75 time of the control in G_1, G_2, G_3, G_4 and G_5 respectively, at the highest NaCl concentration while, K^+ concentration decreased to 0.33, 0.50, 0.64, 0.56 and 0.40 time of the control at the same NaCl concentration (Fig. 4 and 5). Thus, the accumulation of Na^+ under salt induced stress and the leakage of K^+ under both stresses were greater in the stress-sensitive (G_1 and G_5) than in stress-resistant (G_3 and G_2) genotypes. The mannitol-induced osmotic stress decreased the Na^+ concentrations to 60, 40, 55, 42 and 54% of the control in the presence of the highest mannitol concentration, while a decrease of K^+ concentration among the genotypes was reached to about 50, 58, 46, 52 and 54% of the control in G_1, G_2, G_3, G_4 and G_5 respectively (Fig. 4 and 5).

Free proline accumulation

Proline concentration increased significantly and proportionally with an increase in both NaCl and mannitol concentration in the medium (Fig. 6). However, NaCl and mannitol induced stress had different effects on proline accumulation in safflower genotypes. Therefore, the salt treated calli accumulated proline at less extent than the mannitol treated ones. The accumulation differed significantly among the genotypes. Thus, at the highest mannitol concentration, proline accumulation increased by 3.6, 1.8, 2.6, 2.1 and almost 2 fold in comparison with control while it increased about 2.6, 1.4, 2.4, 1.9 which was respectively equal to 2.1 fold in G_1, G_2, G_3, G_4 and G_5 calli in

Table 1. Analysis of variance (ANOVA) for physiological traits of five genotypes calli subjected to salt and drought stress generated by NaCl and mannitol.

S.O.V	DF	Mean squares					
		Proline	sodium	potassium	RGR	RWC	Cell viability
NaCl stress							
Medium (M)	3	2565**	5455**	2351.8**	2.88**	99.6**	98.79**
Genotype (G)	4	781.6**	156.6**	320.4**	0198**	43.17**	25.87**
M× G	12	423.25**	101.9**	28.35**	0.026**	0.84**	11.88**
Error	40	6.34	3.01	6.47	0.009	0.27	0.78
CV%		4.09	5.11	5.67	18.92	5.30	9.95
Mannitol stress							
Medium (M)	3	188089**	130.20**	3737.60**	12.14**	264.90**	109.02**
Genotype (G)	4	6136.1**	76.80**	130.59**	1.69**	49. 70**	35.90**
M× G	12	313.9**	4.30**	13.98*	0. 28**	4. 40**	6.56**
Error	64	33.3	0.15	7.22	0.024	0.24	0.108
CV%		7.68	5.22	6.04	26.57	5.04	4.05

*. **= significant at the 0.05 and 0.01 probability levels respectively; S.O.V. = source of variance; RGR = relative growth rate; RWC = relative water content; CV= Cell viability.

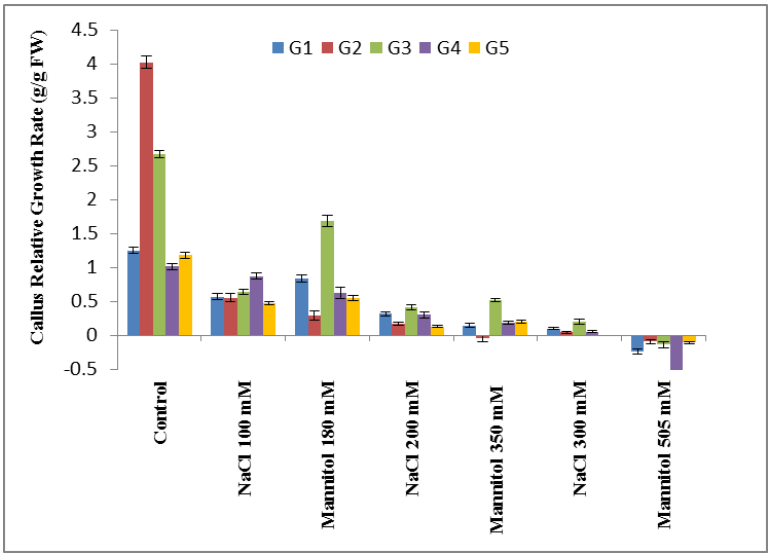

Fig 1. Effect of NaCl and mannitol induced stress on callus relative growth rate of Safflower (*Carthamus tinctorius* L.) genotypes G_1 (LRV-51-51). G_2 (Lesaf). G_3 (Gila). G_4 (Kino-76) and G_5 (Isfahan) calli after 4 weeks of exposure to stress. Each value is the mean of six replicates and vertical bars represent ± standard error.

comparison to the control under salt iso-osmotic stress (Fig. 6).

Cell viability

NaCl and mannitol significantly reduced cell viability of all genotypes compared to the control. Moreover, greater reduction in cell viability was obtained in mannitol treated calli compared to NaCl treated ones. Also, a differential effect was recorded among the genotypes. Cell viability reduction corresponded to 29, 65, 43, 20 and 85% of the control at the highest concentration of mannitol and to 47, 51, 23, 50 and 64% of the control in the presence of the NaCl iso-osmotic concentration in G_1, G_2, G_3, G_4 and G_5, respectively (Fig. 7).

Discussion

The application of NaCl and mannitol induced stress showed a considerable decrease in both RGR and water content values among all genotypes. However, a highly significant difference was recorded among the effects triggered by each kind of stress. The highest RGR and water content decrease were noticed under mannitol-induced osmotic stress. These finding indicated that RGR inhibition might be due to the water availability reduction and the loss of turgor in both G_2 and G_4 genotypes, which also was reported previously in species such as *Carthamus tinctorius* (Zebarjadi et al., 2010), *Oryza sativa* (Lutts et al., 1996), *Triticum durum* (Bajji et al., 2000; Lutts et al., 2004), *Tagetes minuta* (Mohamed et al., 2000) and *Saccharum sp*. (Errabi et al., 2007). In contrast, G_1 and G_3 seemed to preserve a higher RGR value under mannitol-induced osmotic stress. These findings allow us to conclude that at the plant cell level, G_2 and G_4 is more drought resistance. Under salt-induced osmotic stress, G_3 is considered as salt-resistant genotype, while G_5 is relatively salt-sensitive genotype. These results corroborate with those obtained at the whole plant level in relation to salt stress (Karimi et al., 2011). A decline in callus growth upon salt stress may be because of the nutritional imbalance due to an interference of salt ions, such as Na^+ and Cl^- with essential nutrients involved in both uptake and translocation processes

Table 2. Mean comparison of physiological traits of five genotypes calli subjected to salt and drought stress generated by NaCl and mannitol.

	Cell viab. (g^{-1}FW)	Tol. Index	RGR (g g^{-1}FW)	RWC %	Proline (µM g^{-1}DW)	Potassium (µM g^{-1}DW)	Sodium (µM g^{-1}DW)
NaCl stress							
G_1	8.09^{b}	0.26^{b}	0.32^{b}	8.04^{d}	69.22^{b}	314.11^{d}	444.16^{a}
G_2	7.49^{c}	0.04^{d}	0.25^{c}	9.71^{b}	76.41^{a}	359.02^{c}	398.24^{c}
G_3	8.74^{a}	0.15^{c}	0.41^{a}	10.44^{a}	60.37^{c}	433.39^{a}	338.03^{d}
G_4	5.36^{d}	0.40^{a}	0.40^{a}	10.52^{a}	59.83^{d}	$424.43a^{b}$	427.69^{b}
G_5	8.08^{b}	0.16^{c}	0.19^{d}	9.22^{c}	58.42^{d}	411.57^{b}	441.95^{a}
Mannitol stress							
G_1	10.93^{a}	0.19^{b}	0.24^{b}	8.36^{c}	97.40^{b}	356.75^{e}	93.47^{a}
G_2	7.63^{b}	0.008^{d}	0.05^{d}	7.08^{d}	105.98^{a}	397.14^{c}	74.47^{c}
G_3	7.09^{b}	0.25^{a}	0.68^{a}	12.02^{a}	69.13^{c}	373.40^{d}	$76.79b^{c}$
G_4	7.05^{b}	0.09^{c}	0.1^{c}	9.03^{b}	68.25^{c}	403.84^{b}	36.25^{d}
G_5	4.24^{c}	0.18^{b}	0.22^{b}	11.85^{a}	69.13^{c}	456.25^{a}	78.93^{b}

Means followed by the same letter(s) in each column are not significantly different (Duncan's Multiple Range Test 5%). Cell viab. = Cell viability. Tol Index= Tolerance Index. RGR = relative growth rate; RWC = relative water content

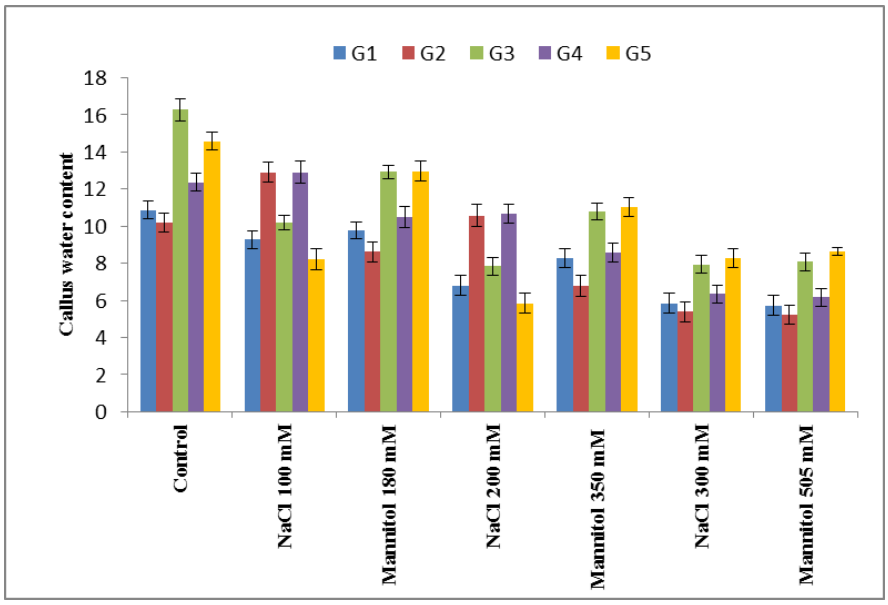

Fig 2. Changes in callus water content of Safflower (*Carthamus tinctorius* L.) genotypes G_1 (LRV-51-51). G_2 (Lesaf). G_3 (Gila). G_4 (Kino-76) and G_5 (Isfahan) calli as affected by NaCl and mannitol induced stress after 4 weeks of exposure to stress. Each value is the mean of six replicates and vertical bars represent ± standard error.

(Errabi et al., 2007). The fact that NaCl affected the RGR value at lesser extent is mostly might be due to the selective accumulation of Na^+ ion. As previous researches also showed, moderate increase of Na^+ and Cl^- within callus tissue might avoid water loss and ensure an economic way to adjust osmotically (Chinnusamy and Zhu 2003; Benlloch-Gonza` lez et al., 2005; Errabi et al., 2007). However, when the ability of the cells to compartmentalize the ions into the vacuole is exceeded, ions build up in the cytoplasm and lead to severe ion imbalances and to conformational changes in the plasma membrane electrical potential (Chinnusamy and Zhu 2003; Sairam and Tygai 2004; Silva et al., 2010). In our work, an expected increase in Na^+ internal concentration of salt-stressed calli was observed (Fig. 4) with significant difference among genotypes which collaborated with previous reports for whole plant (Almansouri et al., 1999). Furtheremore, NaCl-treated calli of G_3 genotype exhibited a high ability to cope with internal Na^+ since were the least affected by the highest dose of salt in terms of both growth and cellular viability. Also, Calli of the salt-resistant G_3 accumulated less Na+ than calli obtained from the salt-sensitive G_5 as was reported in sugarcane (*Saccharum sp*.) (Errabi et al., 2007) and rice (Lutts et al., 1996). Although, low accumulation of Na^+ in the salt-resistant genotypes of wheat varieties suggest that these genotypes have developed an exclusion mechanism to cope with the presence of salt in the medium (Trivedi et al., 1991). In contrast, high accumulation of Na^+ in the salt sensitive genotypes could be due to the lack of efficient compartmentation of Na^+ ions, which build up in the cytoplasm until reaching critical levels (Chinnusamy and Zhu 2003). However, the attribution of the deleterious effects of salt stress solely to the Na^+ toxicity might be an oversimplification of the events taking place during the exposure to salinity (Lutts et al., 1996). K^+ is a major cation in cell organization and it was reported to be a major contributor to osmotic adjustment under stress conditions in several species (Santos-Diaz and Alejo-Ochoa 1994; Bajji et al., 2000; Ghasempour et al., 2007). Under salt-induced stress, the increase in Na^+ concentration lead to decrease in K^+ concentration among safflower genotypes which this trend reported in several other species previously (Basu et al., 2002; Benlloch-Gonza` lez et al., 2005; Errabi et

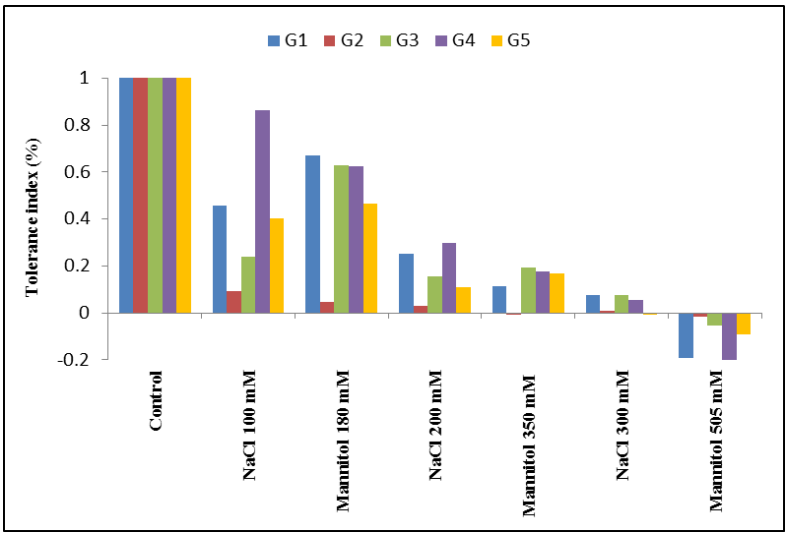

Fig 3. Effect of NaCl and mannitol induced stress on tolerance index of Safflower (*Carthamus tinctorius* L.) genotypes G_1 (LRV-51-51). G_2 (Lesaf). G_3 (Gila). G_4 (Kino-76) and G_5 (Isfahan) calli after 4 weeks of exposure to stress. Each value is the mean of six replicates and vertical bars represent ± standard error.

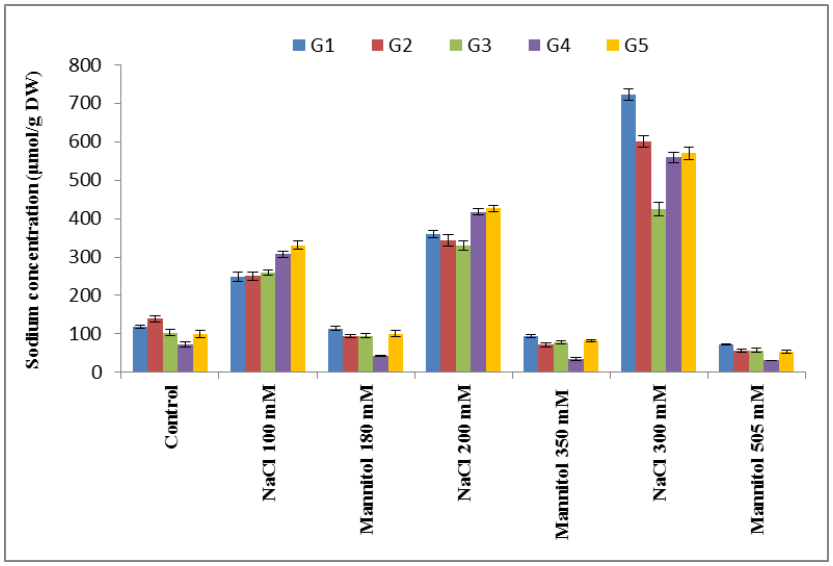

Fig 4. Effect of NaCl and mannitol induced stress on sodium concentration in Safflower (*Carthamus tinctorius* L.) genotypes G_1 (LRV-51-51). G_2 (Lesaf). G_3 (Gila). G_4 (Kino-76) and G_5 (Isfahan) calli after 4 weeks of exposure to stress. Each value is the mean of six replicates and vertical bars represent ± standard error.

al., 2007; Patade et al., 2008). Therefore, the obtained results are in agreement with the previous findings that some species were able to substitute K^+ by Na^+ to ensure the osmotic adjustment (Rus et al., 1999). Over results indicated that, non-ionic induced osmotic stress (mannitol), a substantial decrease of K^+ and Na^+ were recorded among the genotypes. Similar results were reported earlier in mannitol-treated safflower (Zebarjadi et al., 2010) wheat (Trivedi et al., 1991), rice (Lutts et al., 1996) and sugarcane callus (Errabi et al., 2007). Furthermore, the impact of mannitol on plant K^+ tissue was significantly differed depending on treated genotypes. Even though, the decreased K^+ concentration which was monitored suggests that this cation is not involved in osmotic adjustment of mannitol-treated calli obtained from the safflower genotypes. In summary, our results indicated clearly that the disruption of the ion concentration occurred under both salt- and mannitol- induced stress. Also, accumulation of some kind of compatible solutes is another strategy that plant adopt to withstand stress conditions (Parida and Das, 2005). Furthermore, Compatible solutes fall into three major groups: amino acids (e.g., proline), quaternary amines (e.g., glycine betaine, dimethylsulfoniopropionate), and polyol/sugars (e.g., mannitol, trehalose) (Lutts et al., 2004; Karimi et al., 2011). The accumulation of compatible solutes in the cytosol and organelles, where they function in osmotic adjustment and osmoprotection, is essential for salt tolerance (Hasegawa et al., 2000; Patade et al., 2008). Also, proline is frequently involved in osmotic protection in higher plants, and has been reported to be associated with salt and drought tolerance (Benlloch-Gonza` lez et al., 2005; Errabi et al., 2007; Ghasempour and Kianian, 2007; Patade et al., 2008, Zebarjadi et al., 2010). Ultimately, It was approved that proline have a key role in stabilizating cellular proteins and

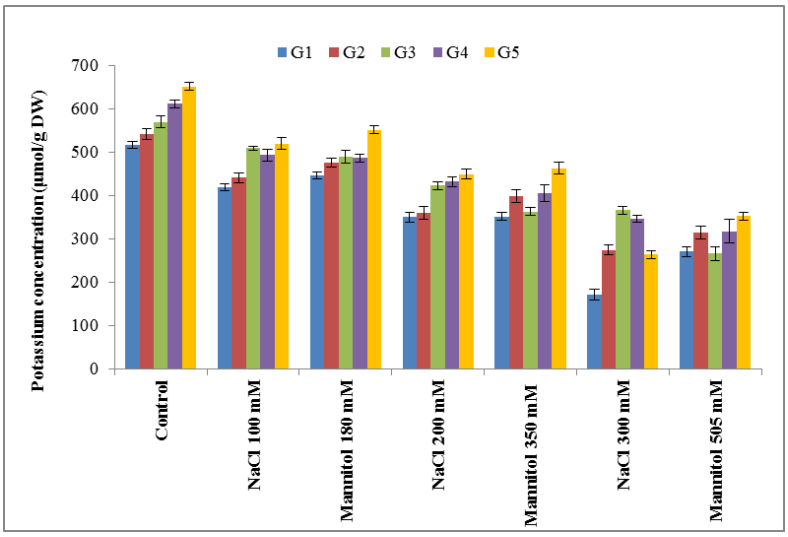

Fig 5. Effect of NaCl and mannitol induced stress on potassium concentration of Safflower (*Carthamus tinctorius* L.) genotypes G_1 (LRV-51-51). G_2 (Lesaf). G_3 (Gila). G_4 (Kino-76) and G_5 (Isfahan) calli after 4 weeks of exposure to stress. Each value is the mean of six replicates and vertical bars represent ± standard error.

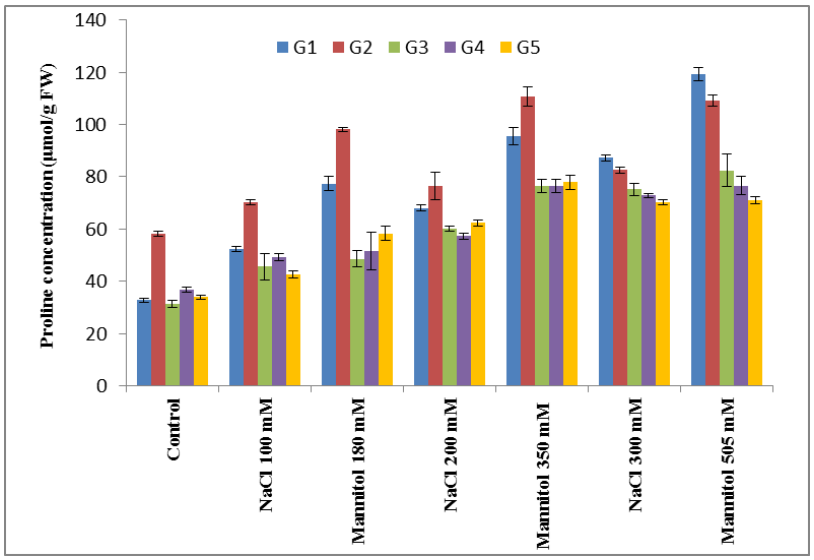

Fig 6. Changes in proline concentration of Safflower (*Carthamus tinctorius* L.) genotypes G_1 (LRV-51-51). G_2 (Lesaf). G_3 (Gila). G_4 (Kino-76) and G_5 (Isfahan) calli as affected by NaCl and mannitol induced stress after 4 weeks of exposure to stress. Each value is the mean of six replicates and vertical bars represent ± standard error.

membranes in presence of high concentrations of osmoticum (Errabi et al., 2007). Other reports indicated that, in safflower genotypes, proline concentration increased considerably under salt- and mannitol-induced stress, which shows that the overproduction of this compound is a non-specific response (Bajji et al., 2000; Alvarez et al., 2003; Ashraf and Harris 2004; Mutlu and Bozcuk, 2005; Errabi et al., 2007). Our results affirmed that, Mannitol induced- stress had higher effect on proline accumulation. Thus, the greatest accumulation of proline is thought to be due to the osmotic component of drought stress (Ashraf and Harris, 2004). The results of this study revealed that under both stresses, the stress-resistant genotypes accumulated proline at higher extent than the stress-sensitive one. These findings suggest that proline is a stress resistance marker. Zebarjadi et al., (2010) showed that there was an almost linear positive relationship between proline accumulations with increasing concentrations of mannitol in the medium of safflower calli which approved the results of this study. Identical statements were reported in several other species (Alvarez et al., 2003; Ehsanpour and Fatahian, 2003). Also, proline can serve as an organic nitrogen reserve ready to be used after stress relief to sustain both amino acid and protein synthesis (Trotel et al., 1996; Sairam and Tygai, 2004).This experiment showed that, reduction in cell viability at 300 mM NaCl was about 46, 51, 33, 49 and 64% of the control in G_1, G_2, G_3, G_4 and G_5, respectively. The decreased cell viability in salt-stressed calli may be associated with toxic effects of increased Na^+ and reduced K^+ contents (Karimi et al., 2011). From a comparative point of view, when compared to unstressed controls, the impact of mannitol on cell viability reduction was higher than salt induced osmotic stress. A reduction in cell viability was also reported in safflower (Zebarjadi et al., 2010), tobacco (Watad et al., 1991), durum wheat (Lutts et-

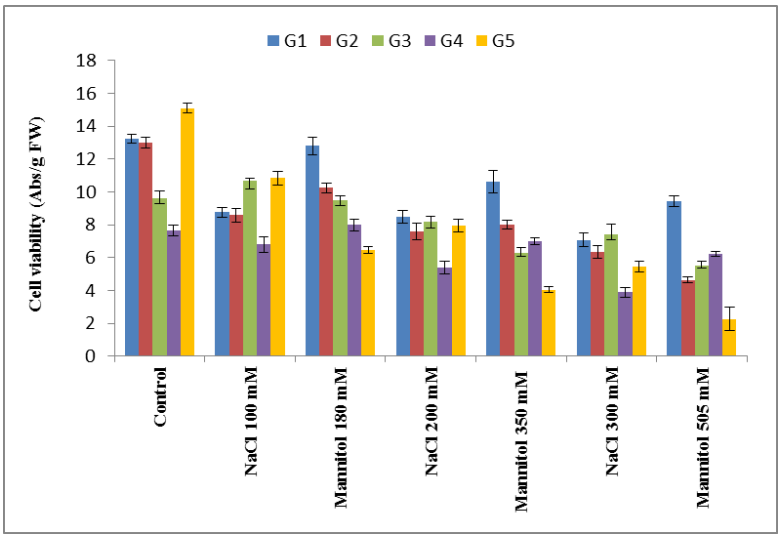

Fig 7. Effect of NaCl and mannitol induced stress on cell viability in Safflower (*Carthamus tinctorius* L.) genotypes G_1 (LRV-51-51). G_2 (Lesaf). G_3 (Gila). G_4 (Kino-76) and G_5 (Isfahan) calli after 4 weeks of exposure to stress. Each value is the mean of six replicates and vertical bars represent ± standard error.

al., 2004) and Sugarcane (Patade et al., 2008) in response to salt and drought stress.

Material and methods

Plant materials

Seeds of *C. tinctorius* five genotypes (G_1, LRV-51-51; G_2, Lesaf; G_3, Gila; G_4, Kino-76; G_5, Isfahan) were obtained from the experimental farm of the Faculty of Agriculture, Razi University, Iran in September 2010.

Seed germination and callus induction

Seeds of safflower were surface sterilized in 20% (v/v) sodium hypochlorite for 20 min, followed by 3 washes with sterile distilled water under aseptic condition. Seeds were sowed directly on MS (Murashige & Skoog 1962) medium with 2% sucrose and 0.7% agar. For callus induction, small segments (5 × 5mm) of cotyledons were aseptically cultured on Murashige and Skoog's (1962) medium supplemented with sucrose (2%), agar (0.7%) and growth regulators- α-naphtaleneacetic acid (NAA), (0.5 mg l^{-1}) in combination with BAP (0.5 mg l^{-1}) and 2,4-dichlorophenoxyacetic acid (2, 4-D), (0.5 mg l^{-1}). All the cultures were maintained at 25±1°C under 16 h illuminations (70 µmolm^{-2} s^{-1}) and 55–60% relative humidity. The pH of the medium was adjusted to 5.7 prior to autoclaving at 121 °C for 15 min. The cultures were monitored for 4 weeks and subcultured for successive three times to study their growth potential and regeneration capacity.

Invitro NaCl and Mannitol stress

After 4 weeks, calli of uniform size (diameter ranking from 7 to 10 mm) were individually weighed and placed on MS medium supplemented with stressing agents: NaCl (100, 200 or 300 mM) and mannitol (180, 350 and 505 mM). These various iso-osmotic concentrations corresponded to osmotic potentials of −0.78, −1.24 and −1.69 MPa determined by a vapour pressure Wescor 5500 osmometer. The control medium had an osmotic potential of −0.32 MPa. For each treatment (genotypes × osmotic agent × osmotic potential), 30 calli were weighed and further characterized for physiological parameters. After four weeks, the calli were rinsed with distilled water several times to remove the adhering ions, the moisture was removed by blotting and fresh mass was taken.

Callus growth

Callus relative growth rate (RGR) was determined on a fresh weight (FW) basis using the formula (FWf − FWi)/FWi where; FWf and FWi are the final and initial fresh weight of the calli. To compare cultivar-related responses to stress conditions, a tolerance index (TI), based on RGR, was calculated according to the following formula: TI = $RGR_{treatment}/RGR_{control}$

Determination of relative water content (RWC) in callus

Callus water content was calculated as a percentage of fresh mass (Al-khayri and Al-bahrany 2004). Dry mass was determined after drying the callus at 60°C for 48 h to a constant mass. The callus water content was calculated as (Fresh weight – dry weight)/dry weight.

Na^+ and K^+ analyses

Na^+ and K^+ contents of the calli treated with or without mannitol and NaCl were assayed by flame photometry method. 0.1 g of each dried calli was digested in 2 ml 3% Sulfosalicylic acid solution (SSA). After complete digestion of the sample, the final volume was diluted with distilled water. Then, the sodium and potassium content of each sample was measured by flame photometer.

Determination of cell viability

For analysis of cell viability, the TTC method (Lutts et al., 2004) was applied. 50 mg calli were rinsed in deionized water containing 0.05% Tween-20 and then incubated at 30°C in the dark in tubes containing 5.0 ml of 0.5% 2,3,5-triphenyltetrazolium chloride in 50 mM K_2HPO_4 (pH 7.0) for 15 h. The samples were then filtered and washed with distilled water, incubated in 3 ml of 94% ethanol at 80°C for

5 min under gentle agitation (80 rpm) to ensure homogenization during the extraction. Samples were then centrifuged at 5,000 × g for 1 min, and absorbance of the supernatant of each sample was measured at 487 nm by spectrophotometer (Tomas 302, USA). The viability index was defined as the absorbance measured g-1 FW.

Extraction and determination of proline

Free proline content was determined according to Bates *et al*., (1973). 500 mg of fresh callus tissue was homogenized in 3% (w/v) sulphosalycylic acid and homogenate filtered through filter paper. After addition of acid ninhydrin and glacial acetic acid, resulting mixture was heated at 100°C for 1 h in water bath. Reaction was then stopped by using ice bath. The mixture was extracted with toluene, and the absorbance of fraction with toluene aspired from liquid phase was read at 520 nm (Tomas 302, USA). The concentration of proline was estimated by referring to a standard curve prepared using L-proline.

Statistical analysis

This experiment was laid out in a completely randomized design (CRD) with three replications. Data were analyzed statistically by the SPSS 16. One-way ANOVA was performed to test the significant differences for all measurable variables. Duncan's multiple range test (DMRT) was performed to compare among the groups for significant differences.

Conclusion

The present study highlights the importance of the effects of both ionic and osmotic component of the salt stress on safflower callus. These results suggested that the physiological mechanisms which mediate the response to salt and drought stress are different. It is noteworthy that growth retardation and reduced cell viability were associated with a loss of turgor under mannitol-induced stress and conspicuous increase in Na^+ but a corresponding decline in K^+ concentrations, demonstrating a typical glycophytic nature of safflower. Moreover, ion status in safflower calli is closely related to the nature of the stress factor applied in the medium. Our results suggest that the accumulation of salt ions (Na^+ and K^+), osmolytes (proline) and retention of an adequate water status may have an important role in osmotic adjustment in safflower genotypes under salt and mannitol stress. Also, our results revealed that G_3 (Gila) can be considered as relatively drought and salt resistance genotype.

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