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The effects of salt stress on physio-biochemical traits, total phenolic and mucilage content of *Plantago ovata* Forsk under *in vitro* conditions

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Summary

Plantago ovata Forsk (psyllium) is an important source of mucilage which is an ingredient in certain drugs and foodstuffs. The calli of 14 genotypes of psyllium were cultured on MS (MURASHIGE and SKOOG, 1962) medium containing 0, 100, and 200 mM NaCl, for four weeks, and the effects of salt stress on the following callus traits were evaluated: growth rate (CGR), relative growth rate (RGR), relative water content (RWC), Na^+ concentration, K^+ concentration, K^+/Na^+ ratio, proline content, total phenolic compounds (TPC), and mucilage content. A reducing trend was observed in GR, RGR, RWC and K^+/Na^+ of the callus cultured in the medium with 100 mM NaCl, comparing to NaCl-free medium, while an increasing trend was observed in Na^+ content, proline content, and TPC under the same conditions. Mucilage content of callus was found to increase in the medium containing 100 mM NaCl (0.13 g g^{-1} DW) but decreased afterwards at 200 mM NaCl (0.117 g g^{-1} DW), albeit with significant variations among genotypes. The results showed that among evaluated genotypes, Isfahan-1 was the most salt tolerant genotype at cellular level. In addition, the highest mucilage content was obtained in Khor-Biabanak genotype when the calli grown at 100 mM NaCl. It was postulated that mucilage content likely to be associated with salt tolerance and could be exploited to counteract the negative osmotic potential in callus affected by salt stress in *P. ovata*.

Keywords: callus induction, medicinal plant, mucilage, psyllium, tissue culture

Introduction

Global agricultural and food production are seriously threatened by high soil salt levels (PARVAIZ and SATYAWATI, 2008; ARZANI and ASHRAF 2016), which affects many of the plant biochemical and physiological processes at the cellular or whole plant level (ASHRAF and HARRIS, 2004; GUPTA and HUANG, 2014; ARZANI and ASHRAF, 2016). Plants' response to salt stress comprises numerous processes in cells that coordinately operate to alleviate hyperosmolarity and re-establish ionic homeostatic conditions (ARZANI and ASHRAF, 2016). Development of salt tolerance in plants is, hence, a complex phenomenon (RAI et al., 2011). Saline-induced osmotic stress triggers a wide range of perturbations ranging from growth and development disruption to modification of the ion transport and uptake systems (BASU et al., 2002; PARIHAR et al., 2015). Moreover, intracellular solutes and certain inorganic ions like K^+ and Na^+ play intrinsic roles in plant response to salt stress (BASU et al., 2002; ARZANI and ASHRAF, 2016). The synthesis and accumulation of compatible osmolyte such as proline is one of the effective mechanisms of adaptation to salt stress by stabilizing membranes and macromolecular structures (ASHRAF and FOOLAD, 2007), detoxifying reactive oxygen species, and adjusting cellular osmosis (ZHU, 2002).To this may be added the fact that actively growing cells, like those experiencing salt stress, may be characterized by a high phenolic content (LIM et al.,

2012). Moreover, many studies have shown that changes in the levels of secondary metabolites, including phenolic compounds, enhance plant defense mechanisms against stress, particularly against oxidative stress induced by high salt concentrations (MATKOWSKI, 2008; LIM et al., 2012).

The study of salt tolerance mechanism is preferably conducted at the cellular level because it provides the means to study plant physiological and genetic processes independently of the regulatory mechanisms occurring at the whole plant level (ARZANI, 2008; LOKHANDE et al., 2010; SHIBLI et al., 2011). The *in vitro* salt stress has been studied in such different species as rice (BASU et al., 2002), *Medicago sativa* (EHSANPOUR and FATAHIAN, 2003), wheat (ARZANI, 2008), Cucurbitacea (CANO et al., 1998; SHIBLI et al., 2011), date palm (AL-KHAIRY, 2002), oil seeds (ALVAREZ et al., 2003; SOHEILIKHAH et al., 2013), sugar cane (GANDONOU et al., 2006), and medicinal plants such as *Foeniculum vulgare* (KHORAMI and SAFARNEJAD, 2011) and *Thymus vulgaris* (ZIA et al., 2010). *Plantago ovata* Forsk, commonly known as psyllium, is an annual medicinal herb plant with beneficial health properties, whose seeds have found important medicinal applications in treating high blood pressure, high cholesterol, diabetes, and hemorroid (SHARMA, 2004). The leaves, seeds, and seed husks of psyllium are rich sources of phytochemical substances such as mucilage (TALUKDER et al., 2015). The plant is native to the Mediterranean region and is found in India, Pakistan, and Iran (SHARMA, 2004). *Plantago* genus is generally considered to be moderately tolerant to salt, though depending on the genotype and salt stress level (KARIMI and HAGHIGHI-PAK, 2012). To the best of the authors' knowledge, no published work has been reported on the biochemical and physiological characterization and mucilage production of psyllium, especially with respect to its mechanisms for combating salt stress at the cellular level. Moreover, the efficient and economical production of mucilage from the psyllium calli through *in vitro* salt stress culture optimization was one of our desired goals. We tested this hypothesis in a series of experiments, in which the types of growth regulators combinations and concentrations have already been tested. Thus, the experiment described here is the final experiment of the series to investigate the physiological and biochemical adaptive mechanisms of different genotypes of psyllium involved in responding to *in vitro* salt stress. In addition, the effects of NaCl treatments on the phenolic and mucilage contents produced as secondary metabolites were assessed at the callus level in response to exogenous elicitor "NaCl".

Materials and methods

Plant material and growth conditions

Fourteen *Plantago ovata* genotypes comprising 12 Iranian (originated from different geographical regions of Iran) and two exotic (from Pakistan and India) genotypes were used in this study. The seeds were obtained from Seed and Plant Improvement Institute, Karaj, Iran. The seeds of 14 genotypes were surface sterilized in 20% (v/v) sodium hypochlorite solution containing 0.1 ml of tween-20 per 100 ml for 15 min, followed by treatment in 95% ethanol for

1-2 min. The Murashige and Skoog (1962) basal salt medium (Sigma-Aldrich) adjusted to pH 5.8, supplemented with 3% sucrose and 0.8% agar (Sigma -Aldrich), and then autoclaved for 20 min at 121 °C and 1.1 Kg cm^{-2}. The sterilized seeds were place on the medium and incubated in a growth chamber in the 16/8 day/night photoperiod at a temperature of 25±2 °C for up to two weeks until full germination.

Callus induction and in vitro salt stress treatment

The calli were induced from hypocotyl explants cultured in MS medium (Sigma- Aldrich) that supplemented with 3% sucrose, 0.8% agar (w/v), 2,4D (0.5) mgL^{-1} and Kin (1) mgL^{-1}. The hypocotyl derived calli were incubated at 24±2 °C for 4 weeks under dark conditions. Then, the calli were transferred onto solid MS medium containing the same concentrations of growth regulators as above and different concentrations of NaCl: 0 (control), 100 and 200 (mM) [0, 0.6 and 1.2% NaCl (w/v)] for 4 weeks (Rai et al., 2011). The cultures were transferred to fresh medium every 2 weeks. After 4 weeks of salt treatment, different physiological and biochemical traits were measured.

Physiological traits

Relative growth rate (RGR) of callus was calculated as $(W_1-W_0)/W_0 \times 100$, where W_0 is the initial callus fresh weight and W_1 was considered as the final fresh weight of callus after 4 weeks of salt treatment (Errabii et al., 2007). Relative water content (RWC) was calculated as [(callus fresh weight – callus dry weight)/callus dry weight $\times 100$] (Lutts et al., 2004). Callus samples of known fresh weight were dried in an oven set at 65 °C for 48 h, after which they were re-weighted and the difference in the initial and final mass determined. The callus growth rate (CGR) was calculated by measuring callus growth (mm/day) at 0, 15 and 30 days after callus transferring to salt containing media induction according to Compton (1994). The callus diameter (di) was calculated by root square of (callus length × callus width) (Compton, 1994).

Biochemical traits
Ion assay (Na$^+$ and K$^+$)

Flame photometry method was used for measuring sodium and potassium concentration of the treated calli based on the method described by Skoog et al. (2007). First, calli were collected and dried at 60 °C for at least 5 days, then 10 mg of each powder of dried callus was digested with 10 ml 3% (w/v) aqueous sulfosalicylic acid for 24 h at 4 °C, sample extract purified with Whatman No. 1 filter paper and Na$^+$ and K$^+$ concentrations were measured. The standard solutions of NaCl and KCl prepared from reagent grade salts were used to estimate the ions concentrations by flame photometer (Jenway Model PEP7, UK).

Proline content

The modified Bates et al. (1973) method was used for proline determination. In this method, 40 mg of callus tissue was homogenized in 1.7 ml of 3% (w/v) aqueous sulfosalicylic acid. The extractions were transferred to centrifuge tubes and centrifuge at 14000 g for 20 min. Then 1 ml of each supernatant transferred into a 10 ml test tube, 1 ml of glacial acetic acid and 1 ml of the ninhydrin reagent added to each tube and heated for 1 h at 100 °C. Tubes were then cooled using cold water and 2 ml of toluene added to each tube, sealed with aluminum foil and vortexed at 30 rpm for 2 min. The phase separation was completed after 30 min and the upper phase was used for measuring the absorbance at 520 and 490 nm by spectrophotometer (Shimadzu UV-120-02, Japan).

Total phenolic compound

The extracts was prepared from 25 g 4 weeks old friable calli powdered in liquid nitrogen and immediately suspended in 50 ml of methanol and kept at room temperature for 24 hrs with periodic shaking. The solution was then filtered through Whatman filter paper no. 41, the methanolic extract was concentrated until dry. The dried methanolic extract was dissolved in least amount of methanol and kept at refrigerator temperature 2-4 °C till further use (Umesh, 2014). Total soluble phenolic compounds (TPC) were determined by using Folin-Ciocalteu reagent according to the method of Makkar et al. (1997). Aliquots of extract (200 μl) was taken from each sample, and the volume was made up to 1 ml with methanol. Then, 0.5 ml of Folin-Ciocalteu reagent (1:1 with water) and 2.5 ml of sodium carbonate solution (20%) were added sequentially in each tube. After vortexing, all the tubes were incubated at room temperature in the dark for 40 min. Absorbance was measured at 725 nm against the reagent blank. The results are means of three repetitions expressed in the form of gallic acid equivalents (GAE) per gram of dry mass.

Mucilage assay

Mucilage was extracted from callus according to Gupta et al. (2015) with minor modification in psyllium.

Statistical analysis

The experiments were conducted as either completely randomized design (CRD) or factorial experiment based on CRD with five replications for each treatment. Five calli per replication were randomly selected and analyzed for free proline, total phenolic compounds, mucilage content, ion concentrations (Na$^+$ and K$^+$) and physiological traits. The data was analyzed using analysis of variance (ANOVA) by PROC GLM of SAS (SAS Institute, 2011). Mean comparisons were conducted according to Fisher's least significant difference (LSD) test at the 0.05 level of probability. Principal component analysis was performed using PROC FACTOR (method = prin) with, that is, Eigen value ≥ 1.0 in SAS 9.3 (SAS Institute, 2011). Then, a genotype-by-trait biplot was constructed by using the first two trait-focused scaling principal components (PC$_1$ and PC$_2$) derived from principal component analysis (PCA) of a genotype-by-trait matrix containing standardized trait data. A simple regression and a multivariate backward stepwise regression analyses were also performed using PROC REG of SAS version 9.3 (SAS Institute, 2011).

Results and discussion
Physiological traits

The results of analysis of variance showed significant differences among the genotypes investigated and the significant effects of salt treatments on the physiological traits (Tab. 1). The genotype × salinity interaction was not significant for RWC, CGR and RGR (Tab. 1). Addition of NaCl to psyllium callus cultures led to a significant decline in the callus physiological traits RWC, RGR and CGR under *in vitro* salt stress (Fig. 1a, b) in *P. ovata* calli. Karimi and Haghighat-Pak (2012) reported the non-significant reduction of seedling-related traits of psyllium genotypes at 200 mM NaCl under NaCl stress. The inconsistency between the *in vitro* and *in vivo* responses may be explained by the multiplicity of the mechanisms involved in whole plant salt tolerance (Arzani, 2008; Rai et al., 2011). It has been established that the water potential gradient between the cell and the nutrient medium caused by NaCl results in dehydrated cells and reduced callus fresh weight (Al-Khayri, 2002; Rafiq et al., 2008; Lokhande et al., 2010; Shibli et al., 2011) as well as declining callus growth rate (Cano et al., 1998; Ashraf and Ahmad, 2000; Ehsanpour and Fatahian, 2003; Lutts et al., 2004).

Tab. 1: The results of analysis of variance for callus related traits of psyllium genotypes subjected to salinity stress.

Trait[¥]	Genotype (G)	Salinity (S)	G × S	Residual
df:	13	2	26	168
RWC	778.99**	5117.9**	160.03	96.21
CGR	0.93**	14.77**	0.142	0.10
RGR	0.23**	2.39**	0.031	0.021
K$^+$	7222.9**	2492.1**	2182.4**	288.75
Na$^+$	2194.7**	701025**	1459.8	960.6
K$^+$/Na$^+$	1.65**	76.23**	1.12	0.86
Proline	0.047**	18.07**	0.033**	0.005
TPC	6.2**	32.7**	1.31**	0.009
Mucilage	0.003**	0.005**	0.0017*	0.0009

** and * Significant at P < 1% and 5%, respectively.
[¥] df: degree of freedom; RWC: relative water content; CGR: Callus growth rate; RGR: relative growth rate; TPC: total phenolic compound.

RWC decreased significantly with increasing salt stress, but no significant differences were observed in this parameter between the two 100 and 200 mM NaCl levels (Fig. 1a). The changes observed as a result of the reducing relative water content at 100 mM NaCl might have been due to the fast reaction of callus cells to the declining water availability in cells and the resulting loss of turgor pressure (AL-KHAYRI, 2002). Reduced RWC values due to *in vitro* salt stress have been previously reported for different glycophytes (GANDONOU et al., 2006) and halophytes such as *Sesuvium portulacastrum* (LOKHANDE et al., 2010) and *Suaeda nudiflora* (CHERIAN and REDDY, 2003). Callus growth started to be appreciably inhibited at 100 mM NaCl compared to that in the absence of NaCl. The reduced callus growth could be compromised from a reduction in the osmotic potential of the medium culture that enhances the requirement for maintaining the turgor of the growing callus cells, a process that consumes energy and, eventually, leads to the declining callus growth (SHIBLI et al., 2011).

To gain a better understanding of salt-tolerance and salt-sensitivity, some researchers have used the ionic selectivity and osmoprotectant accumulation as traits to classify genotypes regarding their response to salt and also to select tolerant cell lines. The genotypes used in this study showed different physio-biochemical responses to *in vitro* salt stress (Tab. 2). The mean comparisons of the genotypes evaluated

for their callus growth and water related traits showed that salt stress had its greatest inhibitory effect on reducing callus growth in all the genotypes investigated at 200 mM concentration of NaCl (Fig. 2 and Tab. 2). The highest (0.61 mm day^{-1}) and the lowest (-0.19 mm day^{-1}) values of CGR averaged over all the salt levels tested were obtained with Behbahan and Ghazvin genotypes, respectively (Tab. 2). The highest values of RWC (35.55%) and RGR (82%) were obtained with Isfahan-1, whereas the least RWC (11%) and RGR (47%) values belonged to Ghazvin and Isfahan-3, respectively (Tab. 2). On the other hand, the genotypes with sensitive callus had severely reduced callus RWC and RGR under salt treatments relative to the control. Similar results have been reported for other species (BASU et al., 2002; EHSANPOUR and FATAHIAN, 2003; GANDONOU et al., 2006).

RWC was significantly correlated with CGR, RGR and mucilage content under 200 mM NaCl conditions as evident by their vector angles in the biplot presented in Fig. 3. A similar relationship was previously observed between RWC and CGR (BASU et al., 2002; AHMAD et al., 2009). This relationship could be explained by the osmotic effect of NaCl which results in reduction of water content in cytosol causing in turn decline in CGR (ARZANI and ASHRAF, 2016). In addition, the increase in salt concentration may lead to the reduction of osmotic potential of the medium, results in reduced turgor of the growing cells, and eventually leads to a decrease in callus growth (CGR) (HAMEDI et al., 2016).

Biochemical traits
Ion content (K$^+$ and Na$^+$)

K$^+$ ion reportedly plays an important role in enzyme activation (TESTER and DAVENPORT, 2003) although the relationship between K$^+$ content and salt stress may vary from one species to another (AL-KHAYRI, 2002). The results of analysis of variance showed significant differences among the genotypes and the significant effects of salt treatments for ion content (K$^+$, Na$^+$ and K$^+$/Na$^+$) (Tab. 1). The genotype × salt interaction was only significant for K$^+$ concentration (Tab. 1). In the present study, increased salt at 100 and 200 mM (NaCl) led to a significant decrease in K$^+$ concentration (Fig. 2a). Similar to these findings, K$^+$ reportedly declined steadily in response to increasing salt concentration of callus cultures (CHAUHAN and PRATHAPASENAN, 2000; BASU et al., 2002; GANDONOU et al., 2006; LOKHANDE et al., 2010; SOHEILIKHAH et al., 2013). Other studies, however, have reported callus cultures to exhibit an initial increase in their K$^+$ levels in response to low NaCl levels (such as 25 mM) which later declined steadily at higher NaCl levels (AL-KHAYRI, 2002). The reduction in K$^+$ concentration in callus cells under salt stress could be explained by the alterations in expression and/or function of transporters as well as the ion channels especially those related to K$^+$
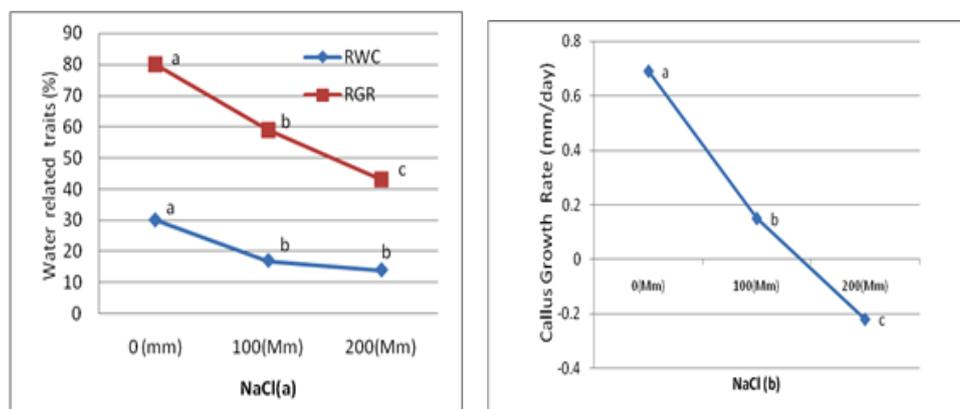
Fig. 1: Effect of *in vitro* NaCl stress on the callus RWC and RGR (**a**) callus growth rate (**b**) of *Plantago ovata*. Means by common letter do not significantly differ at the LSD$_{5\%}$.

Tab. 2: The mean comparisons of different genotypes of *P. ovata* for different physio-biochemical traits and mucilage content under *in vitro* salt stress.

NaCl (mM) Genotype	RWC (%)* Avg.	CGR (mm day⁻¹) Avg.	RGR (%) Avg.	Na⁺ (mmol g⁻¹ DW) Avg.	K⁺ (mmol g⁻¹ DW) 0	K⁺ 100	K⁺ 200	K⁺/Na⁺ Avg.	Proline (mg g⁻¹ FW) 0	Proline 100	Proline 200	TPC (mg GAEg⁻¹ DW) 0	TPC 100	TPC 200	Mucilage (g g⁻¹ DW) 0	Mucilage 100	Mucilage 200
Isfahan-1	35.55^{a}**	0.29^{b-e}	82^{a}	135.5^{d}	242^{a}	104^{b}	78^{a}	1.84^{ab}	0.05^{cde}	0.59^{a}	1.4^{a}	1.90^{e}	2.10^{f}	2.32^{g}	0.11^{a-d}	0.11^{bc}	0.11^{abc}
Mashhad	22.20^{d}	0.21^{d-g}	75^{ab}	170.6^{a}	208^{bc}	110^{a}	68^{ab}	1.41^{abc}	0.08^{a-e}	0.40^{c}	1.06^{c}	2.00^{de}	2.10^{f}	3.30^{d}	0.14^{a}	0.14^{b}	0.14^{a}
Shiraz	12.34^{ef}	0.05^{fgh}	48^{c}	140.6^{cd}	192^{cde}	80^{cde}	50^{bcd}	1.66^{abc}	0.10^{a-e}	0.50^{b}	1.08^{c}	2.80^{b}	3.8^{a}	4.04^{b}	0.12^{abc}	0.13^{bcd}	0.13^{ab}
Ghazvin	11.0^{f}	-0.19^{i}	53^{c}	142.6^{bcd}	218^{b}	90^{bc}	52^{bcd}	2.47^{a}	0.10^{a-e}	0.50^{b}	0.90^{d}	1.90^{e}	2.50^{e}	3.60^{c}	0.09^{cd}	0.10^{c}	0.10^{bc}
Aran- Bidgol	26.01^{bc}	0.47^{abc}	69^{b}	152.8^{a-d}	152^{h}	80^{cde}	52^{bcd}	1.09^{bc}	0.10^{a-e}	0.50^{b}	1.20^{b}	2.10^{cd}	2.8^{d}	3.04^{e}	0.13^{ab}	0.14^{b}	0.14^{a}
Khor- Biabanak	26.14^{bc}	0.42^{a-d}	50^{c}	158.8^{abc}	104^{i}	74^{c-f}	54^{bcd}	0.97^{bc}	0.13^{abc}	0.40^{c}	1.02^{c}	2.0^{de}	1.9^{g}	2.80^{f}	0.08^{d}	0.20^{a}	0.09^{cd}
Pakistan	18.44^{de}	0.25^{c-f}	50^{c}	165.4^{a}	200^{bcd}	72^{c-f}	56^{bcd}	1.53^{abc}	0.03^{e}	0.40^{c}	1.00^{c}	1.01^{g}	1.08^{i}	4.80^{a}	0.10^{bcd}	0.12^{bc}	0.11^{abc}
Ahvaz	22.63^{bcd}	0.50^{ab}	51^{c}	151.5^{ad}	160^{gh}	70^{def}	38de	1.15^{bc}	0.14^{ab}	0.50^{b}	1.20^{b}	1.40^{f}	1.7^{h}	2.40^{g}	0.10^{bcd}	0.11^{bc}	0.11^{abc}
India	27.40^{b}	0.01^{efg}	51^{c}	141.7^{cd}	124^{i}	64^{ef}	48^{cd}	0.79^{bc}	0.04^{de}	0.40^{c}	1.20^{b}	2.20^{c}	3.08^{b}	4.00^{b}	0.12^{abc}	0.14^{b}	0.13^{ab}
Behbahan	19.14^{cde}	0.61^{a}	76^{ab}	173.2^{a}	108^{i}	56^{f}	28^{e}	0.74^{c}	0.16^{a}	0.40^{c}	1.07^{c}	2.20^{c}	3.81^{a}	4.30^{b}	0.10^{bcd}	0.12^{bc}	0.12^{abc}
Chaharmal- Bakhtiari	13.66^{ef}	0.02^{ghi}	72^{ab}	165.93^{a}	112^{i}	70^{def}	38de	1.01^{bc}	0.07^{b-e}	0.55^{ab}	1.20^{b}	3.00^{a}	3.5^{b}	3.90^{b}	0.11^{a-d}	0.11^{bc}	0.11^{abc}
Isfahan-2	24.45^{bcd}	-0.001^{ghi}	52^{c}	164.8^{ab}	164^{fgh}	88^{bcd}	64^{abc}	0.98^{bc}	0.10^{a-e}	0.50^{b}	0.90^{d}	2.00^{de}	2.9^{d}	3.00^{e}	0.12^{abc}	0.13^{bcd}	0.13^{ab}
Isfahan-3	12.71^{ef}	-0.16^{i}	47^{c}	157.8^{abc}	185^{def}	104^{b}	64^{abc}	1.65^{abc}	0.07^{b-e}	0.40^{c}	0.80^{d}	1.50^{f}	2.4^{e}	2.80^{f}	0.12^{abc}	0.14^{b}	0.13^{ab}
Isfahan-4	13.82^{ef}	0.29^{b-e}	71^{ab}	162.3^{abc}	174^{efg}	84^{cd}	54^{bcd}	1.51^{abc}	0.12^{a-d}	0.40^{c}	1.01^{c}	1.0^{g}	1.1^{i}	2.00^{h}	0.10^{bcd}	0.11^{cb}	0.06^{d}
Grand mean					167.3	81.69	53.14		0.10	0.46	1.07	1.93	2.47	3.30	0.11	0.13	0.12

*Means in each column followed by the same letter are not significantly different at the 5% probability level.
** RWC: Relative water content; CGR: Callus Growth Rate; RGR: Relative growth rate; TPC: Total phenolic compounds.
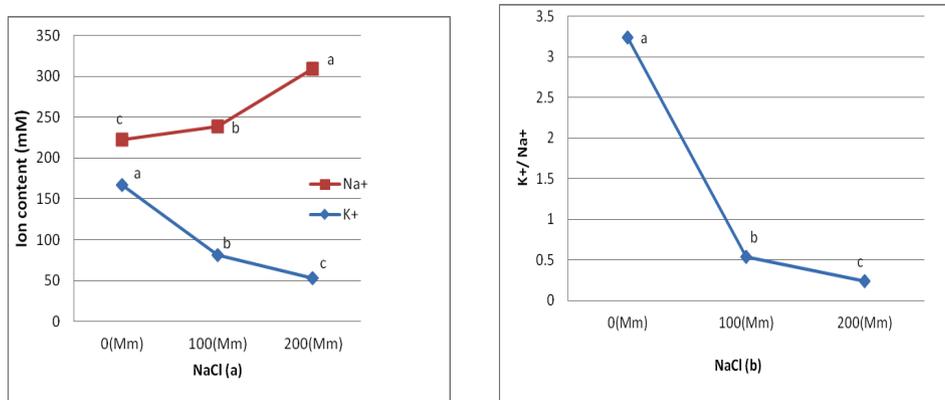
Fig. 2: Effect of *in vitro* NaCl stress on the K$^+$ and Na$^+$ content (a) and K$^+$/Na$^+$ ratio of *Plantago ovata* callus. Mean with one or more letter in common are not significantly different at the 5% probability level as tested by LSD5$_\%$ test.
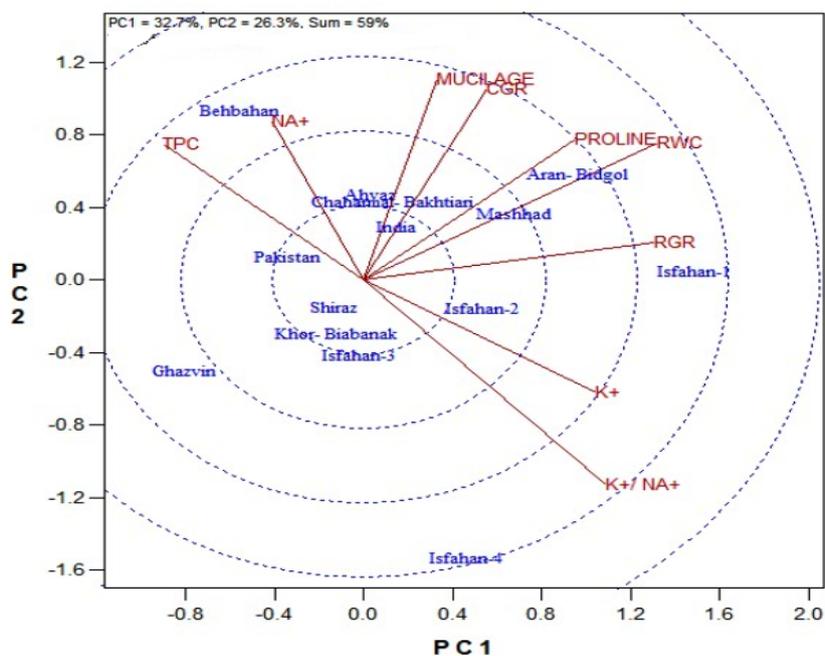
Fig. 3: Biplot drawn based on the first (PC1) and second (PC2) components obtained from principal component analysis using CGR, RWC, RGR, TPC, Mucilage Na$^+$, K$^+$, and K$^+$/Na$^+$ ratio of psyllium genotypes under 200 Mm (NaCl) under *in vitro* conditions.

(ARZANI and ASHRAF, 2016).

The patterns of K$^+$ content in response to increasing NaCl levels (Fig. 2a) were parallel to the trends of callus growth and callus water related traits. For instance, the highest callus growth was achieved with a culture medium containing no NaCl, that is, the same concentration at which the highest K$^+$ uptake was observed. Furthermore, the inhibitory concentration of psyllium callus growth was identified to be 100 mM NaCl (Fig. 1b), which is the same concentration at which potassium concentration significantly reduced relative to the control (Fig. 2a).

Na$^+$ concentration in the callus was observed to increase significantly with increasing salt concentration (Fig. 2a). The sharpest increase in Na$^+$ concentration was observed when the callus was cultured on a medium containing 100 mM NaCl (Fig. 2a). The rising trend of Na$^+$ concentration observed in this study was similar to those reported for such other plants as safflower (SOHEILIKHAH et al., 2013), *Foeniculum vulgare* (KHORAMI and SAFARNEJAD, 2011), and date palm (AL-KHAYRI, 2002). Experimental NaCl concentrations (i.e., 100 and 200 mM) concurrently led to an increase in plant Na$^+$ content (Fig. 2a) although no significant changes were observed in the values

of callus RGR (Fig. 1a). This suggests that the elevated nutritional uptake of both Na$^+$ and K$^+$ might have led to the retention of water in the callus (CHAUDHARY et al., 1997).

Maintaining the cellular K$^+$/Na$^+$ homeostasis is pivotal for plant survival in saline environments (ARZANI and ASHRAF, 2016). As a result of increasing Na$^+$ concentration in the medium, Na$^+$ ions compete with K$^+$ ones during salt stress for the transporter as they both share the same transport mechanisms, thereby decreasing the uptake of K$^+$. Accordingly, the K$^+$/Na$^+$ ratio was observed in this study to decrease significantly from 3.25 (the control) to 0.24 (at 200 mM NaCl) (Fig. 2b). This result is in agreement with those reported elsewhere (CHAUDHARY et al., 1997; AL-KHAYRI et al., 2002). Since the K$^+$/Na$^+$ ratio is critical for salt tolerance, increasing this ratio at 100 mM NaCl in *Plantago ovata* will be a promising area of future research. The genotypes examined were found to differ with respect to their intracellular ions under control conditions (Tab. 1); all the genotypes, however, accumulated more Na$^+$ ions than did the control. The Na$^+$ content averaged over the salt level tested varied from 173.1 (mmol g^{-1} DW) in the Behbahan genotype to 135.5 (mmol g^{-1} DW) in Isfahan-1 (Tab. 2). However, compared to those with salt-sensitive callus

(i.e., Indian and Pakistani), the genotypes with salt-tolerant calli (i.e., Isfahan-1) accumulated less Na^+ but more K^+ ions. The imposition of NaCl-shock caused an injury to the tissue leading to excessive leaching and poor retention of K^+ in the callus in genotypes with lower salt stress tolerance, demonstrating a very sharp reduction in K^+ content with increasing salt from control to higher levels. This confirms that the K^+ inclusion mechanism, which is an indicator of salt tolerance at the whole plant level (KARIMI and HAGHIGHAT-PAK, 2012), was also expressed at the cellular level in psyllium. Furthermore, it is seen that, compared to those with susceptible calli, the genotypes with tolerant calli (such as Isfahan-1) accumulated less Na^+ and maintained higher levels of K^+, rather than the genotypes with sensitive calli (such as Ahvaz). This may be one reason underlying the better growth of the Isfahan-1 calli under NaCl.

The different genotypes and their calli exhibited significant differences in their intracellular ion accumulation (Tab. 2). The evaluated genotypes showed variations in their K^+/Na^+ ratios from 0.74 (in Behbahan) to 2.47 (in Ghazvin) (Tab. 2). The highest K^+/Na^+ ratio recorded for the Ghazvin genotype demonstrated its high salt tolerance and capacity for maintaining an ionic equilibrium between Na^+ and K^+ ions. The harmful effect of Na^+ depends on its accumulation site. Indeed, its accumulation is toxic to salt-sensitive callus in which it is carried into the cytoplasm; it is, however, nontoxic to salt-tolerant callus since it accumulates in their cell vacuoles (VOLKOV, 2015). In this case, the vacuolar sequestration in different genotypes would play an important role in maintaining the water balance in the callus by increasing the osmotic pressure in the cells and keeping a high K^+/Na^+ ratio.

The K^+ content and K^+/Na^+ ratio was found to be correlated with proline content in callus affected by salt stress (Fig. 3). These results were in agreement with those of other researchers who reported a positive and significant correlation between K^+ content with K^+/Na^+ ratio (BASU et al., 2002; EHSANPOUR and FATAHIAN, 2003). According to CHERIAN and REDDY (2003), the reduction in K^+ concentration is capable of inhibiting growth as a result of reducing plant capacity for osmotic adjustment and turgor maintenance, or alternatively, by adversely affecting metabolic functions. In the current study, no significant relationship was found between K^+ and other physiological traits (CGR, RWC and RGR) (Fig. 2). On the other hand, positive relationship was observed between Na^+ with some of these traits. It is expectable that the enhanced Na^+ content would not only disturb plant nutrient balance and osmotic regulation but also reduce growth (GUPTA and HUANG, 2014).

Proline content

Proline acts as osmotica that protects the cytosol from dehydration as symptomatic salt stress damage (ASHRAF and FOOLAD, 2007). The analysis of variance showed significant differences between evaluated genotypes, salt treatments and genotype × salt interaction for proline content (Tab. 1). In psyllium callus, the proline content averaged over all the genotypes was found to increase dramatically with increasing salt from 0.1 mg g^{-1} FW in control to 1.1 mg g^{-1} FW in 200 mM (NaCl) (Tab. 2). The increase in proline content practically occurred at 100 mM NaCl, which indicates that salt stress was problematic to cellular functions at this point. Endogenous, free proline accumulation with increasing medium salt concentration has been corroborated in various *in vitro* culture systems subjected to salt stress (AL-KHAYRI, 2002; EHSANPOUR and FATAHIAN, 2003; ALVAREZ et al., 2003; LOKHANDE et al., 2010). There was a significant difference among the genotypes for proline accumulation, as reported in Tab. 2. Clearly, proline content increased sharply in all the genotypes as the elevated concentration of NaCl. Genotypic differences in proline accumulation under salt stress have also been reported in *Medicago sativa* (EHSANPOUR and FATAHIAN, 2003), safflower

(SOHEILIKHAH et al., 2013).

Proline content did not correlate with CGR, RWC and RGR under 200 mM NaCl conditions (Fig. 2). Although the role of proline accumulation in tolerance to salt stress remains controversial, it is thought play role in osmotic phase of salt stress at either early stages of salt treatment or at mild salt stress conditions (ARZANI, 2008). Nonetheless, some authors reported a negative correlation between proline accumulation and callus growth traits in tomato (CANO et al., 1998) and date palm (AL-KHAYRI, 2002). There was a negative and significant correlation between K^+ and proline content (Fig. 2). It could be explained that proline and K^+ involve in different pathways influencing salt tolerance in plant cells as stated in part in the preceding sentence.

Total phenolic compound

The synthesis and accumulation of polyphenols in *in vitro* cultures are generally stimulated in response to salt stress (KSOURI et al., 2007). The reduction in growth induced by salt stress might have resulted in a new pattern of resource providing additional carbon skeletons for phenolic biosynthesis (KSOURI et al., 2007). The analysis of variance showed significant differences between evaluated genotypes, salt levels and genotype × salt interaction for TPC content (Tab. 1). Total phenolic content increased in term of general mean of the genotypes with increasing salt from control 1.93 (mg GAEg^{-1} DW) to higher levels including 100 mM (2.47 mg GAEg^{-1} DW) and 200 mM (3.3 mg GAEg^{-1} DW) (Tab. 2). These results could be explained by the hypothesis that salt stress might be associated with increasing total phenolic compounds due to antioxidant activities (GIRI et al., 2012; LIM et al., 2012). Elevated phenol contents under salt stress have also been reportedly observed in the callus cultures of other species such as *Salvadora persica* (SHARMA and RAMAWAT, 2014). However, accumulation of phenolic compounds in plants due to salt stress may be species depend (LIM et al., 2012). In addition, it depends on the salt concentrations, i.e. while phenolic compounds has not affected by moderate salt concentration; high salt stress caused a remarkable reduction in TPC in the seedlings of rice (CHUNTHABUREE et al., 2014).

It was found a co-ordinate change in proline content and TPC of callus in this study, while this relationship was not significant (Fig. 3). It has been demonstrated that accumulation of high amounts of proline in the cell increases TPC through the biosynthetic pathway of proline-linked pentose phosphate, which, in turn, enhances the phenyl propanoid synthesis (GIRI et al., 2012).

Mucilage content

The variation in mucilage content was evaluated under *in vitro* salt stress. The analysis of variance showed significant differences between evaluated genotypes, salt levels and genotype × salt interaction for mucilage content (Tab. 1). Grand mean comparison of mucilage content showed slight increase from control (0.11 g g^{-1} DW) to 0.13 g g^{-1} DW at 100 mM NaCl (Tab. 2). The increase in mucilage (as a secondary metabolite) content under salt stress has been reported in such few species as *Salicornia brachiate* (JAISWAR and KAZI, 2016) under *in vivo* conditions. A decrease in mucilage content at 200 mM NaCl (0.115 g g^{-1} DW), rather than 100 mM NaCl (0.13 g g^{-1} DW), may suggest the inhibitory effect of NaCl at 200 mM on *in vitro* mucilage synthesis in psyllium.

No steady trend was observed in the variations of mucilage content in the callus subjected to different salt treatments (Tab. 2). Mucilage content increased in all the genotypes as salt increased from control (0 mM) to 100 mM NaCl and decreased afterward at 200 mM NaCl (Tab. 2). There were significant variations among genotypes for *in vitro* mucilage content of callus grown at control, 100 mM and

200 mM NaCl treatments. The highest mucilage content at 100 mM (NaCl) (0.20 g g^{-1} DW) was produced by Khor-Biabanak genotype. Mucilage is a polysaccharide mixture with highly variable chemical constituents, which may have played an important role in the salt tolerance through modulating water retention and ion homeostasis in plants (Ghanem et al., 2010).

A strong and positive relationship was found between mucilage content and Na$^+$ content affected by 200 mM NaCl at cellular level (Fig. 3). This finding may be a clue to the physiological role of mucilage in salt tolerance of mucilage-containing plants and deserve further investigation. Furthermore, it is in keeping with our goal of the eliciting effects of salt stress on the mucilage content at the cellular level. *In vitro* mucilage extraction and preparation could be an alternative and possible way to produce this medicinal substance.

Conclusions

Substantial variations were detected in the cellular responses of the different genotypes of psyllium to *in vitro* NaCl stress. Although, our data provides evidence of increasing mucilage content under *in vitro* mild salt stress in a mucilage-containing plant, dissecting the physiological and molecular role of mucilage in regulating ion and water status deserves further attentions.

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