



Application of the salt stress to the protoplast cultures of the carrot (*Daucus carota* L.) and evaluation of the response of regenerants to soil salinity

Agnieszka Kielkowska¹ · Ewa Grzebelus¹ · Agnieszka Lis-Krzyżcin² · Katarzyna Maćkowska¹

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Abstract

This is the first study to generate carrot plants for enhanced salinity tolerance using a single-cell in vitro system. Protoplasts of three carrot accessions were exposed to treatment by seven different concentrations of NaCl (10–400 mM). Salt concentrations higher than 50 mM decreased plating efficiency and those of 200–400 mM of NaCl completely arrested mitotic divisions of cultured cells. The protoplast-derived plants from the control and 50–100 mM NaCl treatment were subjected to an 8-week salt stress in greenhouse conditions induced by salinized soil (EC 3 and 6 mS cm⁻¹). 50 mM NaCl stress applied in vitro induced polyploidy among regenerated plants. The regenerants obtained from the 50 and 100 mM NaCl-treated protoplast cultures grown in saline soil had a higher survival rate compared to the regenerants from the control cultures. The salt-stressed plants accumulated anthocyanins in petioles and produced denser hairs on leaves and petioles in comparison to the control plants. Salt stress influenced pollen viability and seed setting of obtained regenerants. The results suggest that salt stress applied in vitro in protoplast cultures creates variation which allows alleviating the negative effects of salt stress on the development and reproduction of the carrot.

Key message

Salt stress applied to carrot protoplasts generates variability manifested in differences in cellular response and variation in ploidy. The adaptation of carrot regenerants to soil salinity was associated with accumulation of anthocyanins and increased hairiness.

Keywords Abiotic stress · NaCl · Protoplasts · Soil salinity · Somatic embryogenesis

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✉ Agnieszka Kielkowska
a.kielkowska@urk.edu.pl

¹ Unit of Genetics, Plant Breeding and Seed Science, Institute of Plant Biology and Biotechnology, Faculty of Biotechnology and Horticulture, University of Agriculture in Krakow, Al. 29-Listopada 54, 31-425 Krakow, Poland

² Unit of Plant Nutrition, Institute of Plant Biology and Biotechnology, Faculty of Biotechnology and Horticulture, University of Agriculture in Krakow, Al. 29-Listopada 54, 31-425 Krakow, Poland

Abbreviations

CPP	Carrot petiole protoplast medium
FDA	Fluorescein diacetate
FW	Fresh weight
MS	Murashige and Skoog medium (1962)
PEM	Proembryonic mass
R	Regeneration medium
TALL	Thin alginate layer

Introduction

Salt-affected soils, are characterized by an excessive amount of soluble salts (i.e. chlorides and sulphates of sodium, calcium and magnesium) and having an effect on growing in it plants. There are two main components of salt stress in plants. Salinity immediately induces osmotic stress and

later, following osmotic response, plants experience ionic stress (Munns and Tester 2008; Shavrukov 2013). Osmotic stress comes from the differences in the osmotic pressure between saline solutes outside the cell and internal solutes within the cells in roots. The osmotic effects of salinity stress cause plasmolysis, inhibited cell expansion and cell death in roots, stems and young leaves, and stomatal closure (Flowers 2004; Kielkowska 2017a, b). The effect of the osmotic stress is manifested in the rapid inhibition of the expansion of young leaves, a decrease in the production of new leaves and reduced stomatal conductance. The second, ion-specific phase is caused by ions (mostly Na^+ and Cl^-), accumulating over time until they reach toxic levels (Mahajan and Tuteja 2005; Hu et al. 2007). Salt stress causes reduction in growth rate and affects photosynthesis (enzymes, chlorophylls, and carotenoids), and also leads to disruption of protein synthesis (Mickelbart et al. 2015). The plant's response to both osmotic and ionic effects of salinity stress triggers enhanced demands on energy resources and energy metabolism; however, production of energy is usually severely reduced under salinity due to the reduction in photosynthetic rate, which can increase the production of reactive oxygen species (ROS) (Chen et al. 2018). For protection against the detrimental effects of salt stress, plants have evolved biochemical and molecular mechanisms. The salt tolerance mechanism in plants comprises sensing and signaling: transcriptomic response to stress, chromatin modifications, epigenetic and post-transcriptional regulation, transport and partitioning of Na^+ , and accumulation of osmolytes (Eryılmaz 2006; Deinlein et al. 2014; Reguera et al. 2014; Mansour and Ali 2017; Golkar and Taghizadeh 2018).

The carrot (*Daucus carota* subsp. *sativus* L.) is one of the most important vegetables worldwide. The global carrot production in 2017 was estimated at 42.8 million tons (combined with the global turnip production). The top world producers of the carrot are China (47.3% of total world production), Uzbekistan, the Russian Federation, the USA, the UK, Ukraine, Poland and Germany (FAO Stats 2017). The carrot is a glycophyte and is classified as a salt-sensitive crop (Shannon and Grieve 1999; Yadav et al. 2011). A decline in root yield (Nagaz et al. 2012) as well as negative changes in total soluble proteins and chlorophyll contents, photosynthetic rate, and enzymes activity both in carrot roots and leaves under salt stress were reported (Gibberd et al. 2002; Bano et al. 2014). Despite the harmful effects of salinity on the carrot, there are regions in Asia where some landraces that withstand increased salt concentrations in the soil were identified (Kasiri et al. 2013); however, the mechanisms of their adaptations are so far unknown.

The culture of the explants on a medium containing biotic/abiotic selective agent(s) for generation of new variability and reflected in survival, regeneration and growth of desired genotypes (somaclonal variants) is defined as in vitro

selection technique (Rai et al. 2011). Some scientists added another aspect to this definition, which requires heritability of somaclonal variation through a sexual cycle. However, it is not always possible to demonstrate heritability of somaclonal variation (Skirvin et al. 1993, 1994; Rai et al. 2011). Variability can be a result of temporary or permanent genetic changes in cells or tissue during in vitro culture. Tissue culture-induced temporary changes are reversible and therefore not heritable; however the regenerants bearing those changes are defined as somaclonal variants (Kaeppeler et al. 2000; Springer and Schmitz 2017). In contrast permanent variants are heritable and often represent an expression of pre-existing variation in the source plant or are due to the *de novo* variation. Although in permanent variants demonstration of heritability of somaclonal variation can also be impossible due to i.e. sterility of regenerants (Phillips et al. 1994). As a result, the causes of somaclonal variation are not always well understood and have not been fully elucidated (Rai et al. 2011). The in vitro selection experiments focused on the induction of tolerance to salt stress in plants uses salts (mostly NaCl in concentrations 200–300 mM in organ or tissue systems) as a selective agent. This approach has been introduced using a number of plant materials (callus, suspension cultures, somatic embryos, shoot cultures, etc.) which has been screened for variation in their ability to tolerate relatively high levels of salt in the culture media. Since first report on *Nicotiana glauca* (Zenk 1974), many attempts have been made to produce salt tolerant plants using in vitro techniques (Zair et al. 2003; Gandonou et al. 2006; Golkar and Taghizadeh 2018; Mozafari et al. 2018). To the best of our knowledge, in vitro selection in carrot was performed only in the presence of polyethylene glycol towards the induction of drought tolerance (Fallon and Phillips 1989). A more recent stage in the development of stress tolerance in plants through in vitro selection was reviewed by Rai et al. (2011).

In this study, for the first time, we applied salt stress to a single-cell in vitro system in the carrot, to generate a population of tissue culture-derived regenerants with respect to their growth in saline soil. We isolated protoplasts of selected carrot accessions and subjected them to salt stress caused by NaCl added to the culture media. The proembryonic mass (PEM) and somatic embryos that developed in vitro under the applied conditions were regenerated into plants. Those plants were then subjected to salt stress in a greenhouse experiment.

Materials and methods

Plant materials

We used three accessions of the carrot (*D. carota* subsp. *sativus* L.), i.e. the open pollinated cv. Dolanka and two

Iranian landraces (DAL and NL) propagated at the Institute of Plant Biology and Biotechnology, University of Agriculture of Krakow, Poland. The seeds of all accessions were surface sterilized in a three-step procedure (Grzebelus and Skop 2014) including incubation in: 40 °C water bath, 0.2% (v/v) solution of fungicide ‘Bravo’ (Syngenta, Waterford, Ireland), 20% (w/v) water solution of chloramin T (sodium *N*-chlorotoluene-4-sulphonamide), 30 min each, and three rinses with sterile distilled water. The seeds were germinated in Petri dishes on solid Murashige and Skoog (MS) medium with vitamins (Murashige and Skoog 1962) supplemented with 30 g·l⁻¹ sucrose and 6.5 g·l⁻¹ plant agar (Biocorp, Poland) and incubated at 26 ± 2 °C in the dark. The emerging seedlings were transferred to glass jars containing hormone-free regeneration medium (R) composed of MS macro- and micro-elements, 0.1 mg l⁻¹ thiamine HCl, 0.1 mg l⁻¹ pyridoxine HCl, 0.5 mg l⁻¹ nicotinic acid, 3.0 mg l⁻¹ glycine, 100 mg l⁻¹ myo-inositol, 20 g l⁻¹ sucrose, and 2.5 g l⁻¹ phytigel (Sigma-Aldrich, St. Louis, MO, USA) and kept in a climate room at 26 ± 2 °C under a 16-h photoperiod and light intensity of 55 μmol m⁻² s⁻¹ (Grzebelus and Skop 2014).

The flow chart showing subsequent steps of the experiment is presented on Fig. 1.

Protoplast isolation and culture

As source material, leaves with petioles of three- to four-week-old *in vitro* grown plants were used and protoplasts were isolated and cultured according to the protocol previously established by us (Grzebelus et al. 2012). Briefly, the leaf tissue collected from 2 to 3 plants into 10 × 1.5 cm glass Petri dish was sliced into small pieces in a 0.5 M mannitol solution and incubated for 0.5 h. Then the protoplasts were released from the tissue during 3-h maceration in enzymatic solution consisting of 1% (w/v) cellulase Onozuka R-10 (Duchefa Biochemie, Haarlem, The Netherlands) and 0.1% (w/v) pectolyase Y-23 (Duchefa), 20 mM 2-(*N*-morpholino) ethanesulfonic acid (MES, Sigma), 5 mM CaCl₂, and 0.6 M mannitol (Sigma), pH 5.6, filter-sterilized (0.22 μm; Millipore, Billerica, MA), on a rotary shaker (30 rpm), and the subsequent round of protoplast purification was performed. The working density of protoplasts was adjusted to 8 × 10⁵ ml⁻¹ using hemocytometer chamber. Later, the protoplasts were embedded in an alginate matrix and a modified procedure of the thin alginate layer technique (TAL) was used, resulting in the final protoplast density at 4 × 10⁵. After polymerization of the alginate matrix on calcium-agar plates the layers with embedded protoplasts were transferred to 4 ml of liquid carrot petiole protoplast (CPP) culture medium (Grzebelus et al. 2012) in 6 × 1.5 cm Petri dishes. The protoplast cultures were incubated in the dark at 26 ± 2 °C for about 8 weeks.

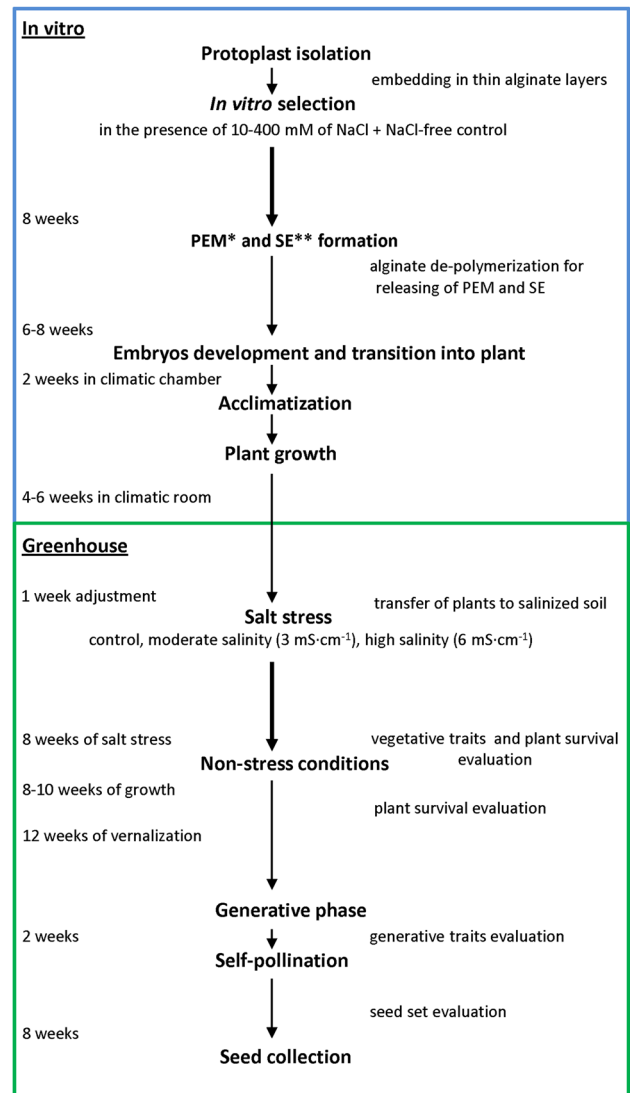


Fig. 1 Flow chart showing subsequent steps of the experiment at tissue culture stage and in the greenhouse. Thick arrows marking the salt stress periods, *PEM proembryonic mass, **SE somatic embryos

The protoplast yield, expressed as a number of protoplasts per gram of fresh weight (FW) of leaf tissue, was determined using the Fuchs Rosenthal counting chamber. The viability of embedded protoplasts was assessed by staining the cultured cells just after immobilization with fluorescein diacetate (FDA) according to Anthony et al. (1999) and presented in percentages. Plating efficiency was expressed as a number of cell aggregates per total number of observed undivided cells and cell aggregates (× 100). All microscopic observations were performed under an Axiovert S100 microscope (Carl Zeiss, Göttingen, Germany) equipped with a filter set appropriate for detecting fluorescein fluorescence ($\lambda_{Ex} = 485$ nm, $\lambda_{Em} = 515$ nm).

Salt stress in protoplast cultures, plant regeneration and acclimatization

The *in vitro* selection was performed from the first day of culture in the presence of NaCl applied to the CPP culture medium in seven concentrations i.e. 10, 25, 50, 100, 200, 300 and 400 mM. CPP medium without NaCl was used as control. The protoplast medium with all supplements, including selective agent, was refreshed once, after 10 days of culture. The proembryonic mass and somatic embryos developed in 8-week-old cultures were released from the alginate matrix by incubation of alginate layers in a sodium citrate solution Grzebelus et al. (2012) and then subjected to regeneration into whole plants on the R medium composed as shown in section "Plant materials". The regeneration procedure was introduced for control and only those NaCl-treated cultures, which showed reduced pattern of development in comparison to control. After two weeks of culture on R medium the number of normal, abnormal (including vitrified) and not regenerating embryos was scored.

About two months after starting the regeneration step, the complete plantlets developed on R medium were transferred to multipots containing 50 ml of coconut substrate (Ceres International Ltd., Pyzdry, Poland) and placed in climatic chambers SANYO MLR-352H (Sanyo Electric Biomedical Co. Ltd., Japan) in 20 °C, with light intensity of 50 $\mu\text{mol m}^{-2} \text{s}^{-1}$, and air humidity of 90%. The plants were acclimatized to *ex vitro* conditions for 2 weeks, by gradually reducing the air humidity to the final value of 70%. Afterwards, the plantlets were replanted to 500 ml pots filled with the AURA substrate for sowing and quilting (Hollas Group, Pasłęk, Poland) and transferred to a climatic room with the temperature of 20 °C and light intensity of 40 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for 4–6 weeks.

During the transfer of the plant material from *in vitro* to *ex vitro* conditions the percentage of acclimatized plants was calculated. For acclimatized plants the ploidy level was evaluated using flow cytometry according to Kiełkowska and Adamus protocol (2010).

The greenhouse experiment

Approximately eight-week-old protoplast-derived plants were transferred to a greenhouse and left there for one week for adjustment. The soil for the experiment was prepared as follow: a top layer of brown soil was mixed with 0.4 g l⁻¹ of Yara Mila™ Complex fertilizer (N-NO₃ + N-NH₄ – 12%, P₂O₅ – 11%, K₂O – 18%, MgO – 2.7%, SO₃ – 20%, B – 0.015%, Fe – 0.2%, Mn 0.02%, Zn – 0.02%, prill granules, Yara International ASA, Norway) and urea 0.1 g l⁻¹, potassium sulfate 0.1 g l⁻¹ and magnesium sulfate 0.6 g l⁻¹. To induce salt stress to plants, the above mineral soil was mixed with 2–4 g l⁻¹ of potassium chloride and 1–3 g l⁻¹

of potassium nitrate. The amount of both latter compounds was determined based on chemical analysis of the soil. The electrical conductivity (EC) was below 2 mS cm⁻¹ for the control (C), and 3 mS cm⁻¹ (moderate salinity) and 6 mS cm⁻¹ (high salinity) respectively for the two salinized soils. The final soil pH was 6.8–7.3. The plants were transplanted to 5 l pots with control soil and with two levels (moderate and high) of soil salinity (Fig. 2h). Pots with the plants were placed on plastic trays and watered daily with 100 ml of tap water (no leachate). Electrical conductivity was determined by measuring the electrical resistance of the soil:water suspension (1:2), according to Camberato et al. (2009). Once a week, soil salinity was analyzed to maintain a stable EC, using a CC-501 conductivity meter (Elmetron, Zabrze, Poland). Salinity was adjusted by application to the soil appropriate amounts (g l⁻¹) of a mixture consisting of 2 g of potassium chloride + 1 g of potassium nitrate for moderately saline soil, and of 4 g of potassium chloride + 3 g of potassium nitrate for high saline soil. Plants were subjected to salt stress for 8 weeks. After this period, the plants were optimally watered and fertilized for undisturbed growth. During spring and summer the plants grew under natural light with the day-length ranging from 14 to 16 h. In the case of plants transferred to the greenhouse later, between October and February, supplemental 16-h lighting provided by sodium lamps (Lucalox™ LU/600W/PSL, GE, Hungary) was introduced. On average, the plants grew at 18 ± 2 °C at night, and 25 ± 3 °C during the day with the relative air humidity (RH) of 65–70%.

At the end of the growing season all the plants which had not flowered earlier, were vernalized for 3 months at 4 ± 2 °C to induce flowering. After vernalization, the plants were transferred to a greenhouse for flowering. At the beginning of flowering, each plant was placed in a net isolator. The umbels containing flowers with receptive stigmas were self-pollinated by hand to produce seeds (Fig. 2l). The seeds were harvested and counted about 8 weeks later.

The mortality of the plants subjected to salt stress was evaluated in two phases: (1) during the salt stress—up to the 8th week of growth in saline soil, and (2) post-stress—since the 10th week after planting to saline soil and later up to vernalization. The plant mortality in the greenhouse experiment was calculated as the number of plants which died per the number of plants planted into the soil in each combination.

At the end of the 8th week of plant growth in saline soil, all the surviving plants were subjected to morphological analyses. The plants were evaluated according to the IPGRI (1998) descriptors for the carrot. The plants were characterized with regard to leaf type and color, leaf growth habit, leaf and petiole length (cm), leaf and petiole hairiness, leaf dissection and anthocyanin coloration of petioles.

In the generative phase, the pattern of flowering (bolters/ flowering after vernalization), the main inflorescence

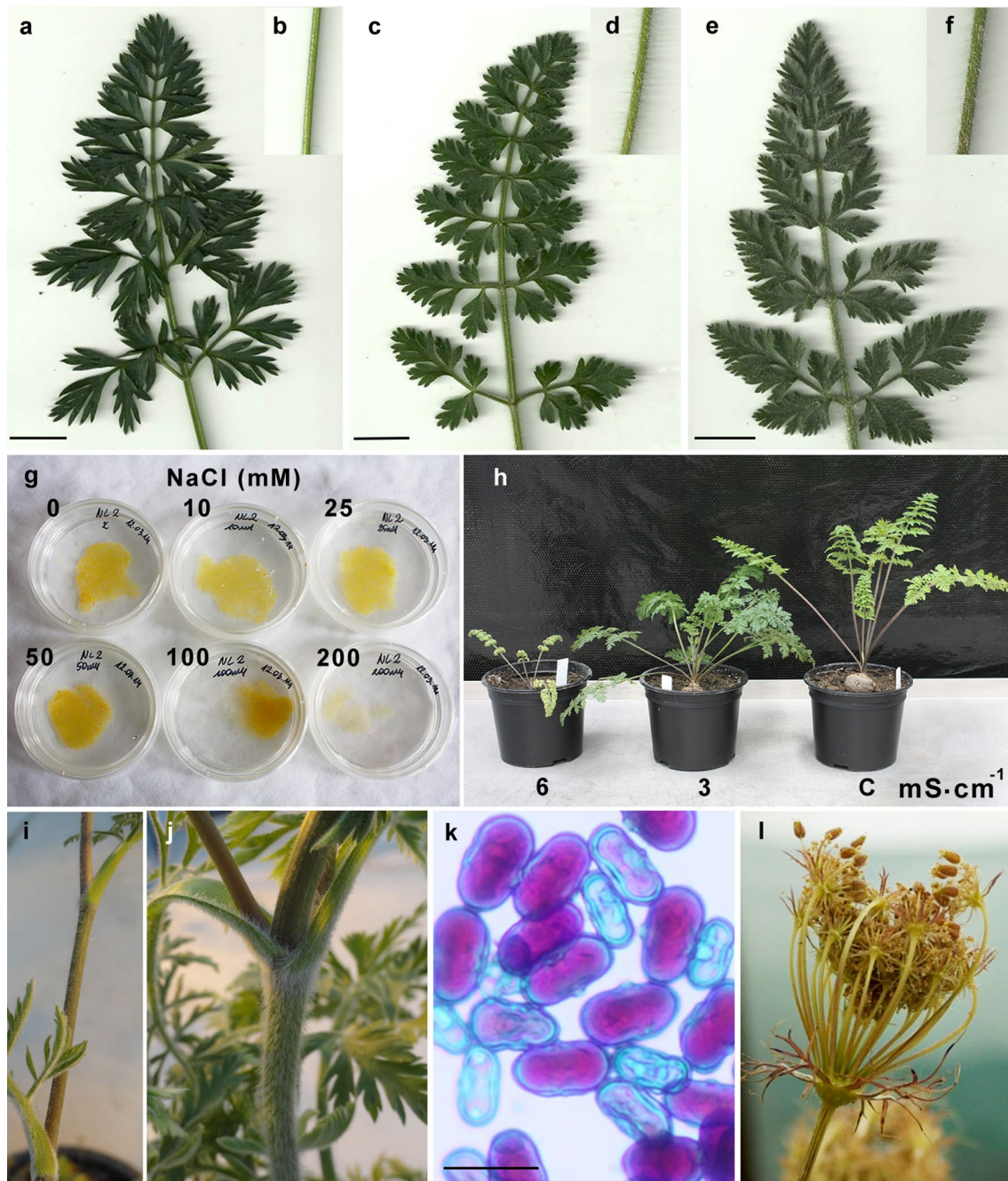


Fig. 2 Application of the salt stress in vitro and the evaluation of the carrot protoplast-derived regenerants exposed to soil salinity. Leaf and petiole morphology of the donor accessions: **a, b** Dolanka, **c, d** DAL landrace, **e, f** NL landrace. **g** In vitro experiment—thin alginate layers overgrown by microcalli colonies of different intensity depending on the NaCl concentration. Greenhouse experiment: **h** carrot

protoplast-derived plants subjected to different levels of soil salinity; **i** stems of Dolanka and **j** DAL landrace grown in highly saline soil (6 mS cm^{-1}) differing in hairiness intensity; **k** pollen viability (magenta-colored viable grains after Alexander staining) and **l** seed setting (after self-pollination) of a salt-stressed carrot regenerant. Scale bars **a, c, e**—1 cm, **k**—20 μm

stem height (cm), the number of umbels, the umbel diameter (cm), and pollen viability was evaluated. The umbel diameter was calculated as the mean of all umbels i.e., the main and the lower order umbels, produced per plant. For viability evaluation, pollen was collected separately from each plant,

stained with Alexander's dye (1969) and observed under a light module of Axiovert S100 microscope. The viable pollen grains were purple, the non-viable light-green (Fig. 2k). Pollen viability was calculated as the number of viable pollen grains per all observed ($\times 100$).

Statistical analyses

In vitro and greenhouse experiments were conducted in complete randomized design (CRD) with at least three replications. For in vitro experiments, each treatment was represented by three Petri dishes and 100–150 cells per Petri dish were observed. The overall effect of treatments was assessed using the analysis of variance (ANOVA) in Statistica ver. 10.0 (StatSoft, Inc., Poland). Tukey's honestly significant difference (HSD) test or the least significance test (LSD) for the in vitro culture or the greenhouse experiments, respectively, were used for mean separation at least at $P \leq 0.05$.

For data covering the selected traits in the generative phase (Fig. 7), statistical grouping was not performed, as in some experimental combinations only single plants flowered, which resulted in a lack of experimental repetitions, therefore for those combinations data were presented as means. For the remaining combinations data were presented as means \pm SE.

The tested accessions subjected to different levels of soil salinity were characterized using principal component analysis (PCA) with the Multivariate Statistical Process Control module in Statistica. Analyze was based on the traits tested in vegetative and generative phase including the pattern of flowering.

Results

Effect of salt stress on protoplast cultures

The description of donor plant material

There were differences in the morphology of seed-derived plants of the tested accessions (Fig. 2a–f). The plants of Dolanka had dark green leaves, with very sparse or no hairiness. The plants of both landraces had denser hairiness on the petioles and leaves. The landraces also had different leaf types. Dolanka had typical, highly dissected leaves, while both landraces had slightly dissected leaves.

The yield and quality of leaf-derived carrot protoplasts

The leaves from in vitro grown plantlets of all accessions have shown to be a very effective source for protoplast isolation, releasing on average $4.5 \pm 0.4 \times 10^6$ cells per g of FW (Table 1). The mean protoplast yield of both Iranian landraces was approximately twice as high (4.8 ± 0.4 and 5.9 ± 0.4 for DAL and NL, respectively) in comparison to our model accession Dolanka ($2.8 \pm 0.3 \times 10^6$, $P \leq 0.01$). The quality of protoplasts embedded in the alginate matrix expressed by the frequency of viable cells after FDA staining was quite high, regardless of the accession, and varied

from $67.6 \pm 1.0\%$ for DAL to $73.6 \pm 1.5\%$ for NL, reaching approximately 70% on average.

The development of the control and NaCl-treated carrot protoplasts

The control protoplasts of all accessions developed as expected i.e. from the fourth day of culture some of them showed symptoms indicating reconstitution of the cell wall such as an increase in size, disappearance of chloroplasts, and reorganization of cytoplasm or a change of the cell shape from spherical to oval. During the following days regular mitotic divisions took place with slightly different frequency depending on the accession, and, as a consequence, cell colonies of various sizes were observed. In early, 10-day-old control cultures the number of cell colonies expressed by plating efficiency varied from 5.3% (NL) to 9.2% (Dolanka). As time passed, that parameter increased from 16.1% (Dolanka) to 18.7% (DAL) in 20-day-old cultures and reached the value between 16.8% (Dolanka) and 24% (DAL) in 40-day-old cultures (Fig. 3). The applied NaCl treatment strongly influenced the development of the cultured protoplast-derived cells ($P \leq 0.01$). A significant decrease in plating efficiency in comparison to the control was observed on the media supplemented with NaCl at the concentration of 50 mM and higher; however, concentrations of 200 mM NaCl and higher completely arrested mitotic divisions as verified during three time-point observations of the culture (Table 2; Figs. 2g, 3).

Regeneration, acclimatization and ploidy of protoplast-derived plants

After two months of culture in the control and in media containing 10–100 mM NaCl, proembryogenic mass was produced. The regeneration procedure was introduced for the control and 50 and 100 mM NaCl-treated cultures only. After release from the alginate matrix, the proembryonic

Table 1 Yield and viability of leaf-derived protoplasts of carrot accessions

Accession	Protoplast yield ($\times 10^6$ /g FW)		Protoplast viability (%)	
	n	Mean \pm SE	n	Mean \pm SE
Dolanka	6	$2.8 \pm 0.3b$	4	67.8 ± 1.6
DAL	5	$4.8 \pm 0.4a$	3	67.6 ± 1.0
NL	6	$5.9 \pm 0.4a$	3	73.6 ± 1.5
Mean/Total	17	4.5 ± 0.4	10	69.5 ± 1.2

FW fresh weight, n number of independent protoplast isolations. Means within columns with the same letters were not significantly different at least at $P \leq 0.05$

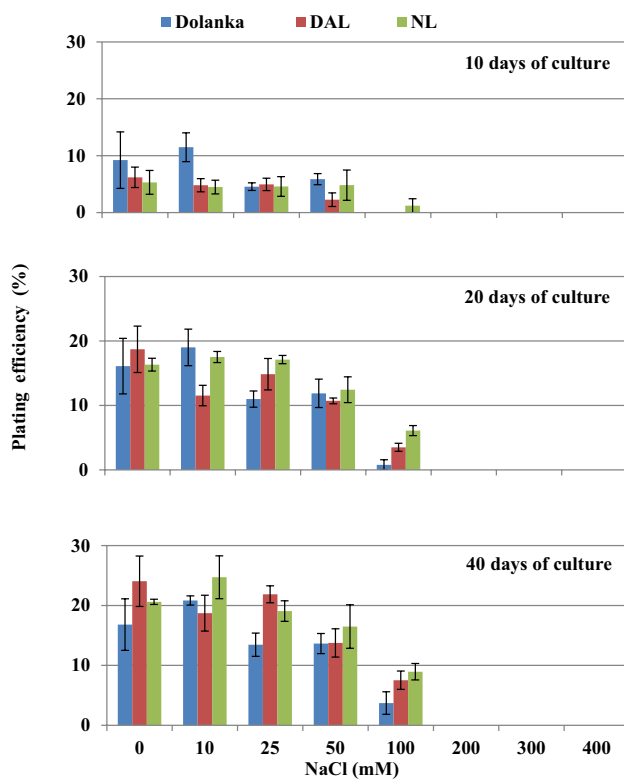


Fig. 3 The effect of NaCl application on plating efficiency in protoplast cultures of different carrot accessions. The bars represent the standard error

Table 2 Mean effect of accession and NaCl concentration on plating efficiency in protoplast cultures of carrot

Treatment	Plating efficiency (%± SE)		
	Age of culture (days)		
	10	20	40
Accession			
Dolanka	3.9±1.1	7.3±1.7	8.6±1.8b
DAL	2.3±0.6	7.4±1.5	10.7±2.1ab
NL	2.5±0.6	8.7±1.6	11.2±2.1a
NaCl concentration (mM)			
0	6.9±1.7a	17.0±1.7a	20.5±2.0a
10	6.9±1.4a	16.0±1.5a	21.4±1.6a
25	4.7±0.6a	14.3±1.2ab	18.1±1.5ab
50	4.3±1.0a	11.7±0.9b	14.6±1.4b
100	0.4±0.4b	3.5±0.9c	6.7±1.1c
200	0.0b	0.0c	0.0d
300	0.0b	0.0c	0.0d
400	0.0b	0.0c	0.0d

Data represent means from three independent experiments (each with three replicates). Means within columns with the same letters were not significantly different at least at $P \leq 0.05$

mass was transferred onto hormone-free R medium and during the following 3–4 weeks transformed into somatic embryos (Table 3). The efficiency of somatic embryo production was negatively affected by the NaCl treatment ($P \leq 0.05$). In comparison to the control, on average, seven- to twenty-five-fold fewer embryos were regenerated from the 50 and 100 mM NaCl-treated cultures (regardless of the accession), respectively (data not shown). Irrespective of the culture treatment, four categories of embryos were observed: normal, vitrified, abnormal with morphological defects and dying (Table 3). The frequency of normal embryos varied from 9% (Dolanka) to 21% (NL) in the control cultures, from 16% (DAL) to 50% (Dolanka) in the 50 mM NaCl-treated cultures and from 0 (NL) to 33% (Dolanka) in the 100 mM NaCl-treated cultures. Within subsequent culture variants from 1 to 88 plants were produced. The regenerated plants from the NaCl-treated and control combinations easily acclimatized to *ex vitro* conditions with approximately 93% efficiency. Most of the analyzed plants were diploids; however, amongst plants regenerated from the 50 mM NaCl-treated cultures, tetraploids were observed (Table 3; Fig. 4).

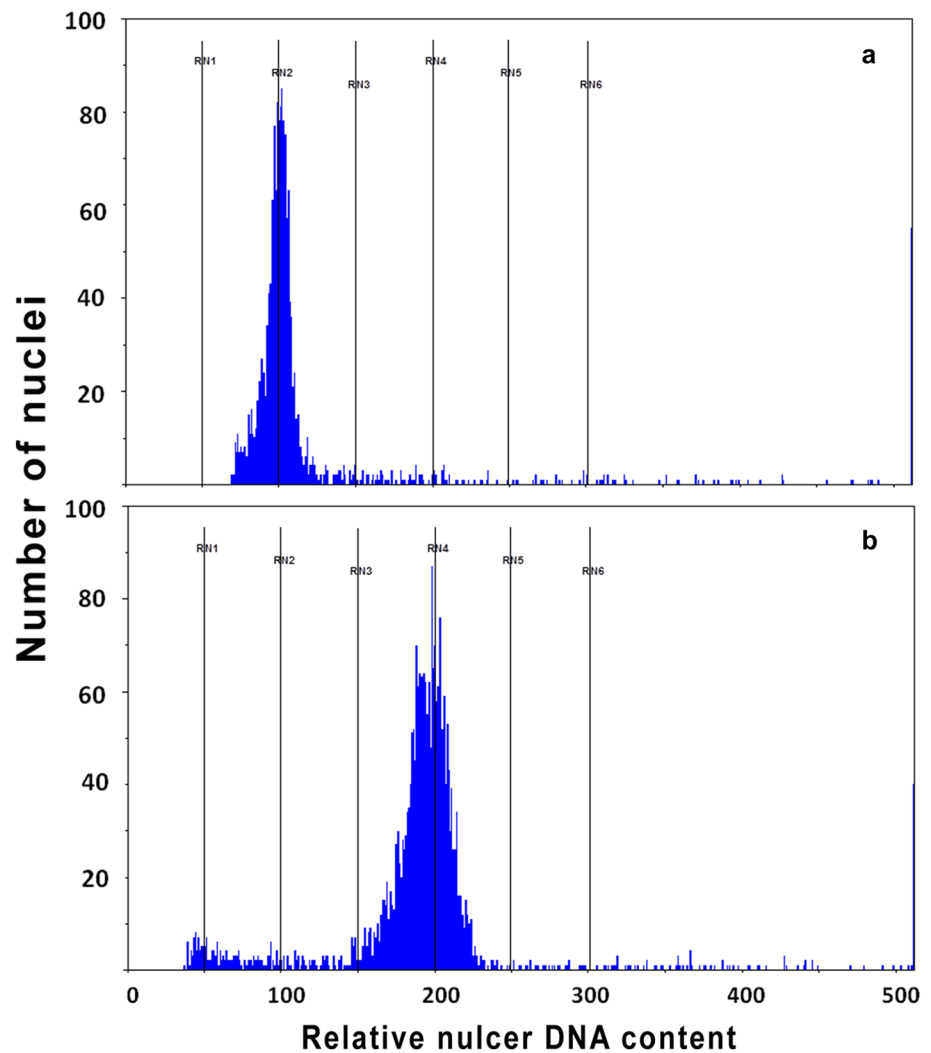
The effect of soil salinity on regenerants obtained from NaCl-treated cultures

Plant mortality

The applied levels of soil salinity affected the survival of protoplast-derived regenerants (Fig. 5). The mortality of the plants increased with the increasing soil salinity, however, differences between accessions were observed. In general, during approximately the first 2–3 weeks of salt stress browning and dying of the oldest leaf was observed while young leaves remained green or yellow-green; this was especially seen in Dolanka and NL. Later, when the salt stress persisted, the leaf growth habit was also affected and changed from erect to semi erect, leaves became yellow–brown and eventually the whole plant died. In general, the highest plant mortality was observed among plants grown in highly saline soil ($P \leq 0.05$). In Dolanka, 74% of the regenerants originating from the control protoplast cultures (0 mM of NaCl) died after planting into the most (EC 6 mS cm^{-1}) saline soil. The mortality of the plants regenerated from the 50 mM NaCl-treated protoplast cultures and subjected to the highest soil salinity was 57%. The majority of plants in this accession died during the salt stress phase (8 weeks), and only 8–13% of the plants died later. Only one plant regenerated from 100 mM NaCl-treated cultures and grown in the most saline soil survived the salt stress period and was later subjected to vernalization. In the DAL landrace the highest (65%) plant mortality was observed among the plants regenerated from 100 mM of NaCl-treated protoplasts and subjected to the highest salinity treatment

Table 3 Characteristic of carrot protoplast-derived somatic embryos from control and NaCl-supplemented media, acclimatization to *ex vitro* conditions and ploidy analysis of regenerated plants

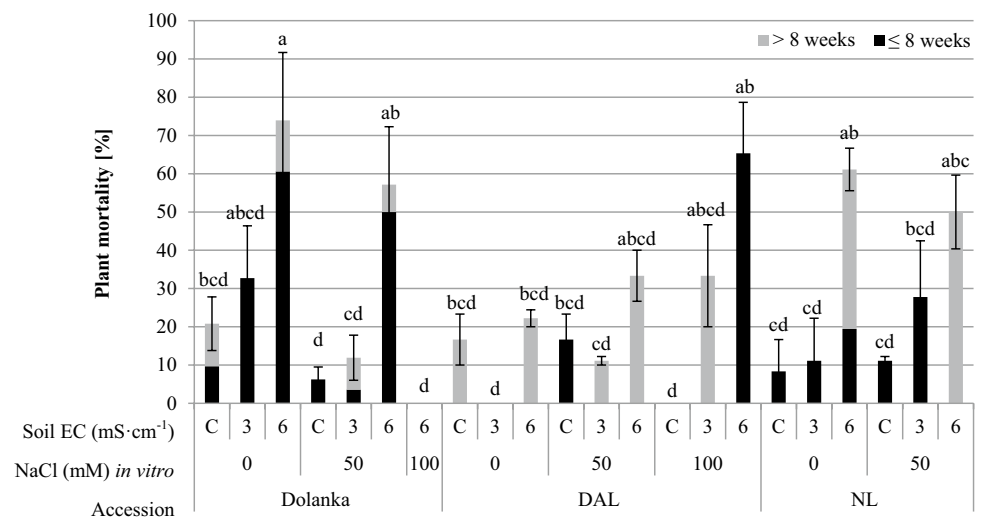
Accession	NaCl (mM)	Somatic embryos					Plant acclimatization (no.)		Ploidy analysis (no.)		
		Total (no.)	Normal (%)	Vitrified (%)	Abnormal (%)	Dead (%)	Regenerated	Acclimatized	Analysed	2×	4×
Dolanka	0	1560	144 (9.2)	1135 (72.8)	275 (17.6)	6 (0.4)	88	79	25	25	
	50	103	51 (49.5)	45 (43.7)	5 (4.9)	2 (1.9)	44	38	17	14	3
	100	3	1 (33.3)	2 (66.7)	0 (0.0)	0 (0.0)	1	1	1	1	
DAL	0	1478	217 (14.7)	559 (37.8)	701 (47.4)	1 (0.1)	26	26	22	22	
	50	196	31 (15.8)	97 (49.5)	68 (34.7)	0 (0.0)	23	21	19	19	
	100	104	20 (19.2)	54 (51.9)	30 (28.8)	0 (0.0)	10	9	8	8	
NL	0	1058	218 (20.6)	331 (31.3)	498 (47.1)	11 (1.0)	31	30	26	26	
	50	336	72 (21.4)	171 (50.9)	90 (26.8)	3 (0.9)	24	23	17	10	7
	100	57	0 (0.0)	16 (28.1)	41 (71.9)	0 (0.0)	–	–	–	–	–

Fig. 4 Flow cytometry histograms showing the relative nuclear DNA content of nuclei isolated from young leaves of NaCl-treated protoplast-derived carrot regenerants: **a** diploid, **b** tetraploid

in the greenhouse experiment. Those plants died during the salt stress phase; however, the remaining plants of this accession died more frequently in the post-stress period. In

the NL landrace the mortality of plants regenerated from the control cultures and grown in the most saline soil was 61%. The plants regenerated from the 50 mM NaCl-treated

Fig. 5 The mortality of protoplast-derived carrot regenerants obtained from NaCl-treated cultures after planting into salinized and control (C) soil. The dark bars represent the percentage of plants which died up to 8 weeks after planting into salinized soil, the grey bars represent plants which died later, up to the vernalization period. The means denoted by the same letter are not significantly different ($p \leq 0.05$, LSD). The presented statistics (letters and \pm SE) jointly represent data for the mortality of plants collected during (≤ 8 weeks) and after (> 8 weeks) salt stress



protoplasts and subjected to the applied soil salinity levels had a higher survival rate. In this accession, the mortality of plants during the salt stress period ranged from 8 to 28% depending on the soil salinity. The mortality of plants in the post-stress period was observed only among the plants grown in highly saline soil.

The analysis of vegetative traits and flowering time

PCA biplots present the graphical data for evaluated traits (vectors) in combinations with accessions and applied treatments (points). The results of PCA analysis showed that the first two principal component axes explained 69.8% of the total variance (Fig. 6, Biplot 1), which allowed description of the differences between the tested accessions. Both Iranian landraces DAL (green-colored, Biplot 1) and NL (blue-colored, Biplot 1) are grouped closely and are located at the negative scores of PC1 and are separated from Dolanka (red-colored, Biplot 1) located at the positive scores of PC1, showing significant differences mainly in leaf type, leaf dissection and time of flowering. Both landraces had slightly dissected leaves of the celery type, while Dolanka had normal, highly dissected leaves (Table S1). PC2 additionally discriminated two landraces from each other, showing that there were differences in leaf color, leaf and petiole hairiness and anthocyanin coloration in the petiole between those accessions (Fig. 6, Biplot 1). The NL landrace had green leaves, sparse hairiness on the leaves and petioles, while the plants of the DAL landrace had gray-green leaf coloration and intermediate to dense leaf and petiole hairiness. Moreover, in this accession, dense hairiness was also observed on the stems (Fig. 2j). The presence of the anthocyanin coloration in the petiole depended on the level of salt stress and, in general, the plants subjected to the highest salinity treatment ($EC\ 6\ mS\ cm^{-1}$) had medium to strongly colored petioles (Table S1).

Since all the accessions were separated into coherent groups, in order to evaluate their reaction to the applied stress conditions we performed PCA analyses separately for each one (Fig. 6, Biplots 2–4). The Dolanka plants grown in the control soil had green petioles, while the plants subjected to increasing salinity in the greenhouse experiment, irrespective of their origin (the control or the NaCl-treated protoplasts) had slight anthocyanin coloration in the petioles (Fig. 6, Biplot 2). The Dolanka plants regenerated from the control protoplast cultures, and subjected to the highest soil salinity and all the plants regenerated from the 50 mM NaCl-treated cultures produced hairs on leaves and petioles. Increased soil salinity caused changes in the leaf color and the length of leaves in the plants regenerated from the control protoplast cultures. The plants subjected to the highest salinity treatment had approximately 8 cm yellow-green leaves, while the plants grown in control soil had green leaves of average length of 21 cm (Table S1). In the plants regenerated from the 50 mM NaCl-treated protoplasts and subjected to increasing soil salinity, no leaf length reduction was observed; however, the highest salinity caused the appearance of yellow-green leaves in 67% of the observed plants. The single regenerant (Dolanka 100-6) obtained from the 100 mM NaCl-treated protoplast cultures and later subjected to the highest salinity was grouped separately from all other Dolanka's experimental combinations but near the DAL landrace (DAL 100-6) originating from the same stress combination *in vitro* (Fig. 6, Biplot 1 and 2). This was mainly due to strong anthocyanin coloration in the petioles and intermediate leaf and petiole hairiness observed in those plants (Fig. 2i). The majority of Dolanka regenerants from the control protoplast cultures and later subjected to the salt stress, flowered typically i.e. after vernalization (Fig. 6, Biplot 2, Table 4). Bolting, in this accession, was noted for 25–33% plants regenerated from the control cultures and subjected to salt stress in the greenhouse experiment as well

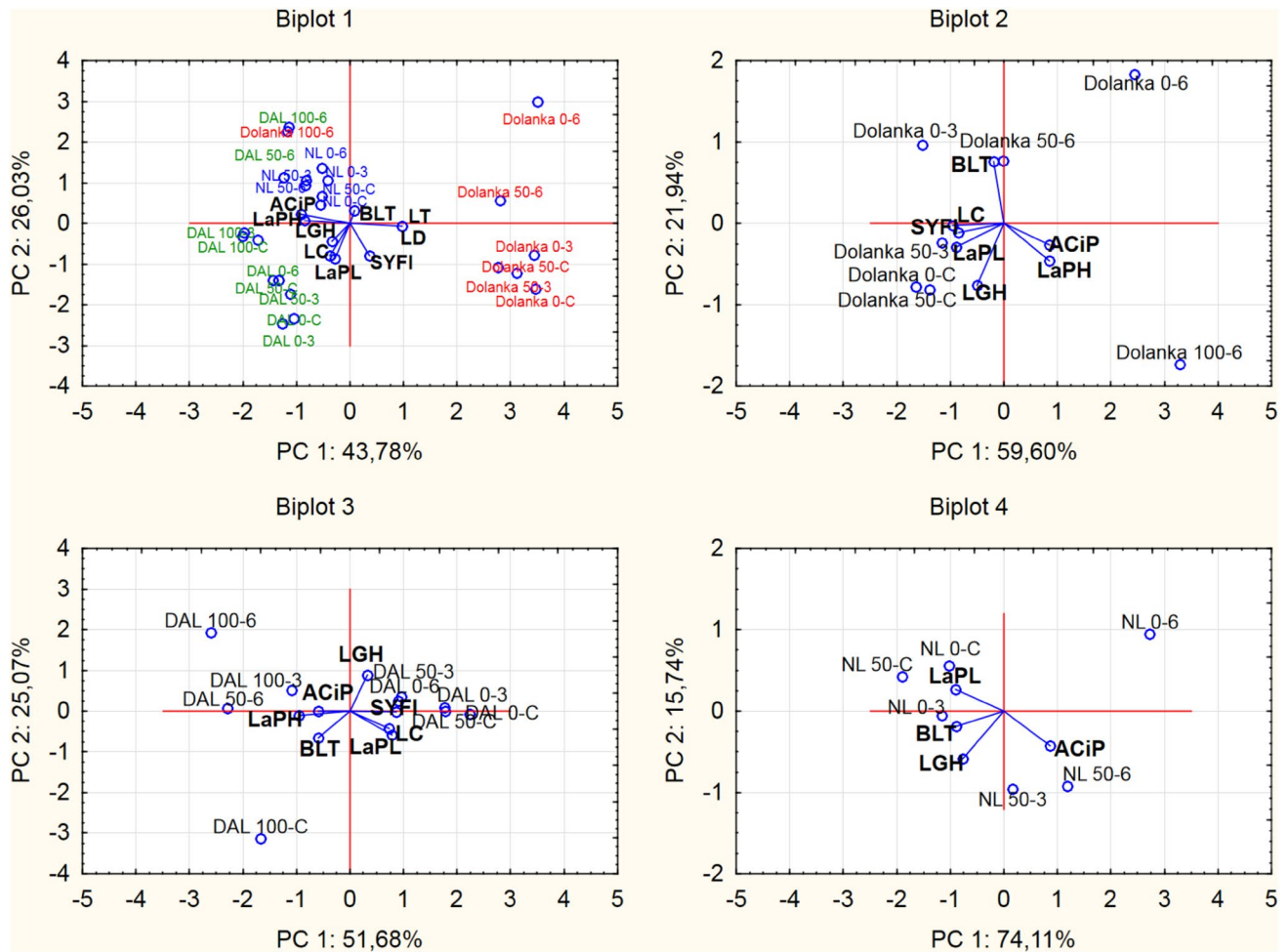


Fig. 6 The principal component analysis (PCA) biplots showing the differences in vegetative traits and the flowering period between the tested accessions subjected to different levels of soil salinity. The observations were made on plants derived from the NaCl-treated protoplasts and then grown for 8 weeks in salinized soil. The analysis also covers stress treatment at the tissue culture stage. Vectors (bolded) represent the analyzed traits: *ACiP* anthocyanin colouration

as in single regenerants from the 50 mM NaCl-treated protoplast cultures grown in moderately saline soil. The sole Dolanka plant regenerated from 100 mM NaCl-treated protoplasts did not survive the vernalization period.

The DAL plants grown in control soil, irrespective of the NaCl treatment during the *in vitro* culture, had longer (30–33 cm) leaves compared to the plants grown in saline soil (21–27 cm) (Fig. 6, Biplot 3, Table S1). All plants grown in saline soil had strong anthocyanin coloration in the petioles, while the plants grown in control soil had intermediate coloration. The plants regenerated from the 50 and 100 mM NaCl-treated protoplasts and later grown in saline soil (EC 3 and 6 mS cm⁻¹) exhibited dense leaf and petiole hairiness (Fig. 2j). The majority of plants of this accession, regardless of soil salinity, flowered after vernalization; with

in petiole, *LGH* leaf growth habit, *LaPH* leaf and petiole hairiness, *LT* leaf type, *LD* leaf dissection, *LC* leaf colour, *LaPL* leaf and petiole length, *BLT* flowering without vernalization, *SYFI* flowering after vernalization. The points represent the following: Dolanka, DAL and NL—accessions names; 0, 50 and 100—NaCl concentration (mM) in protoplast culture medium; C (control), 3 and 6—soil EC value (mS cm⁻¹)

the exception of the two plants regenerated from the 100 mM NaCl-treated cultures, which were bolters (Table 4).

The plants of the NL landrace grown in the control soil, irrespective of NaCl treatment during protoplast cultures, had longer leaves (24–26 cm), compared to salt-stressed plants (17–21 cm) (Fig. 6, Biplot 4, Table S1). The plants regenerated from the 50 mM NaCl-treated protoplast cultures and then subjected to salt stress had strong anthocyanin coloration of the petioles, while the plants grown in control soil had intermediate coloration. All plants of this accession entered the flowering phase without vernalization (Table 4). The plants regenerated from the protoplasts cultured on a NaCl-free medium and then grown in highly saline soil had altered leaf growth habit and did not flower even after vernalization.

Table 4 Effect of soil salinity stress on flowering time and seed production in carrot plants derived from control and NaCl-treated protoplast cultures

Accession	Salinity treatment		Flowering plants no. (%)			No. of plants producing seeds		No. of seeds total/ per plant	
	In vitro (mM NaCl)	Soil EC (mS cm ⁻¹)	Total	BLT	SYFI	BLT	SYFI	BLT	SYFI
Dolanka	0	C	8	0 (0.0)	8 (100.0)	–	4	–/–	308/77
	0	3	8	2 (25.0)	6 (75.0)	0	2	0/0	16/8
	0	6	3	1 (33.3)	2 (66.7)	0	2	0/0	18/9
	50	C	7	0 (0.0)	7 (100.0)	–	3	–/–	115/38
	50	3	7	1 (14.3)	6 (85.7)	0	4	0/0	100/25
	50	6	4	0 (0.0)	4 (100.0)	–	0	–/–	0/0
	100	6	0	–	–	–	–	–/–	–/–
DAL	0	C	5	0 (0.0)	5 (100.0)	–	2	–/–	512/256
	0	3	7	0 (0.0)	7 (100.0)	–	5	–/–	792/158
	0	6	4	0 (0.0)	4 (100.0)	–	3	–/–	587/196
	50	C	3	0 (0.0)	3 (100.0)	–	3	–/–	330/110
	50	3	5	0 (0.0)	5 (100.0)	–	5	–/–	604/121
	50	6	2	0 (0.0)	2 (100.0)	–	1	–/–	59/59
	100	C	1	1 (100.0)	0 (0.0)	–	0	–/–	0/0
	100	3	0	–	–	–	–	–/–	–/–
NL	0	C	2	2 (100.0)	–	2	–	137/69	–/–
	0	3	3	3 (100.0)	–	3	–	230/77	–/–
	0	6	0	–	–	–	–	–/–	–/–
	50	C	4	4 (100.0)	–	3	–	24/8	–/–
	50	3	3	3 (100.0)	–	1	–	18/18	–/–
	50	6	1	1 (100.0)	–	1	–	8/8	–/–
	100	6	1	1 (100.0)	–	0	–	0/0	–/–

C control soil, BLT plants flowered without vernalization, SYFI plants flowered after vernalization

The analysis of generative traits and seed yield

All flowering plants of the tested accessions produced the main inflorescence stem terminating with the primary umbel, and 1–4 lateral inflorescence stems terminating with umbels of higher order. The inflorescence was a compound umbel comprising several umbellets. The longest main inflorescence stems (109–133 cm) were observed in the DAL landrace irrespective of soil salinity (Fig. 7). In the Dolanka plants grown in the control soil, the main inflorescence stem had 112 cm on average, while the plants subjected to saline soil produced shorter (78–90 cm) stems. The Dolanka plants grown in saline soil, regardless of NaCl treatment *in vitro*, produced more umbels per single plant (average 2–3) than the control plants (1–2); however, a nominal decrease in their size was observed. The average diameter of the umbels of the Dolanka plants grown in control soil ranged from 7 to 8 cm, while the umbels of salt-stressed plants had a diameter of 5–7 cm. In both tested landraces, the number of umbels produced by a single plant increased with the increasing soil salinity, with the plants subjected to the highest salinity treatment producing 4 umbels per plant on average, while

the average result in control conditions was 2 umbels per plant. In most cases, the plants of both landraces produced larger umbels (6–12 cm) compared to Dolanka (5–8 cm).

The accessions differed in pollen viability depending on the stage-point of the salt stress application: *in vitro* protoplasts or in the greenhouse on the regenerated plants (Fig. 7). The Dolanka plants regenerated from the control protoplast cultures and then grown in the control soil had relatively high (84%) pollen viability, while plants subjected to salt stress had 68–77% of viable pollens. In the plants regenerated from the 50 mM NaCl-treated protoplast cultures, pollen viability was strongly affected. The Dolanka plants grown in moderately saline soil had on average 38% of viable pollen, while those grown in highly saline soil produced unviable pollen grains. The pollen viability of the DAL plants regenerated from the 50 mM NaCl-treated protoplast cultures was very high (93–96%) irrespective of soil salinity. In the plants regenerated from the control protoplast cultures and subjected to salt stress in the soil, pollen viability ranged from 40 to 77%. The only plant regenerated from the 100 mM NaCl-treated protoplast cultures and later grown in control soil had 44% of viable pollen; however, the

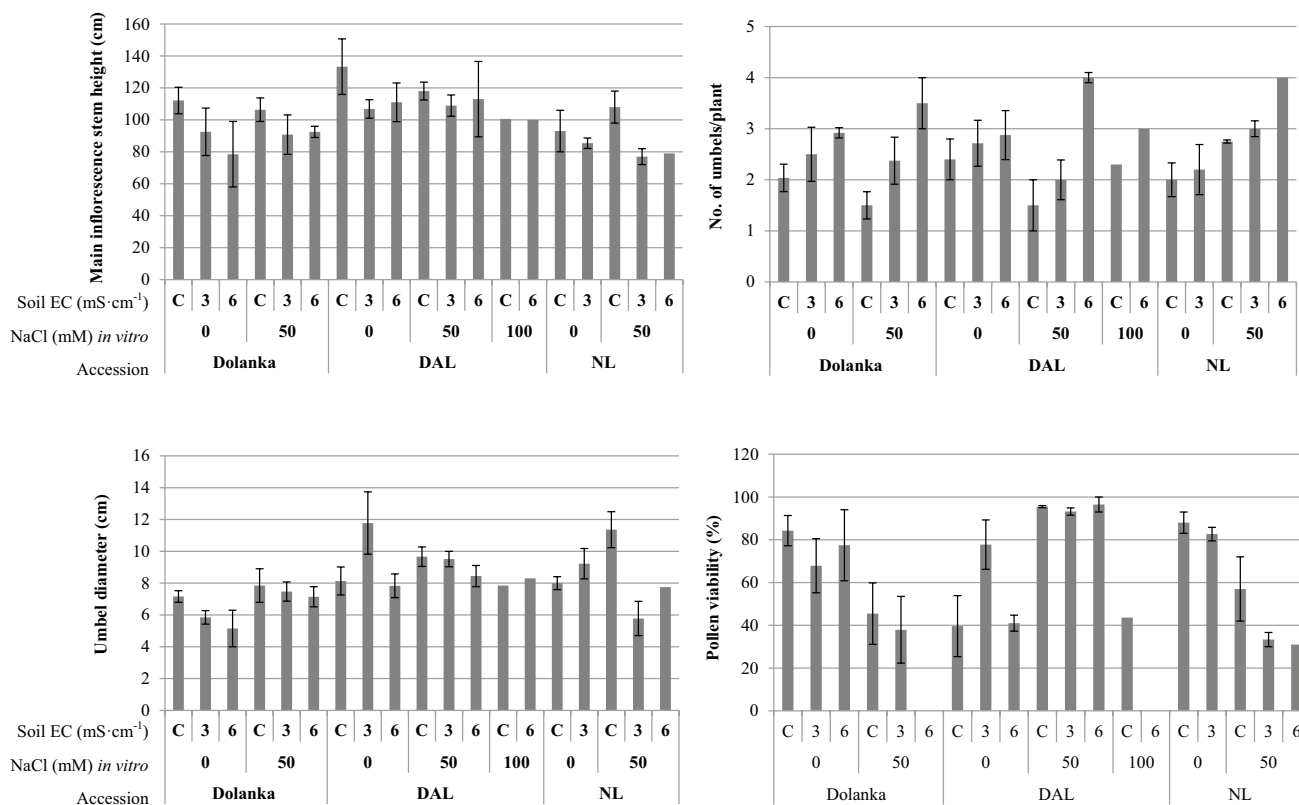


Fig. 7 The effect of salinity on the selected inflorescence features and pollen viability in the carrot. C the control

other plant of the same origin but grown in highly saline soil produced unviable pollen. Regardless of soil salinity, both those plants flowered without vernalization, unlike all the remaining plants in this accession. In the NL plants regenerated from the control protoplast cultures no differences in pollen viability were observed between those grown in the control (88%) and moderately saline soil (83%); however, the plants grown in the most saline soil did not flower. The plants regenerated from the 50 mM NaCl-treated protoplast cultures and grown in saline soil had low pollen viability (31–33%).

In general, the seed yield was negatively affected by soil salinity, however, differences were observed depending on the accession and the NaCl-treatment *in vitro* (Table 4). The highest numbers of seeds produced after self-pollination of the salt-stressed and control plants were observed in the DAL landrace. The plants regenerated from control protoplast cultures and grown in control soil produced on average 256 seeds per plant, but seed production was also observed in plants grown in saline soil. The plants regenerated from the 50 mM NaCl-treated protoplasts and grown in control or in moderately saline soil produced a similar number of seeds (110–121/plant), however, when grown and flowering in highly saline soil, they produced merely 59 seeds per plant on average. The plants regenerated from 100 mM

NaCl-treated cultures did not produce seeds. In Dolanka, the highest number of seeds (77/plant) was obtained from control protoplast-derived plants (0 mM NaCl) and grown in control soil, however, for plants of the same origin, grown in saline soil, the seed yield was significantly decreased (8–9 seeds/plant). The plants derived from the 50 mM NaCl-treated cultures and grown in control and moderately saline soil produced on average 25–38 seeds per plant, while plants grown and flowering in highly saline soil did not produce seeds. The Dolanka plants grown in saline soil flowered without vernalization, mostly developed single umbels and did not produce seeds. A higher seed yield (69–77/plant) in NL was observed in the control protoplast-derived plants. Single plants regenerated from the 50 mM NaCl-treated cultures produced roughly 8–18 seeds per plant.

Discussion

Considering the assessment of salt tolerance in tissue cultures, the issue of cellular vs. whole-plant response is of great importance, therefore in our study we used the protoplast system to generate variation within three carrot accessions, and later subjected the regenerants to soil salt stress to evaluate the whole-plant response. Application

of protoplasts as source material to produce biotic/abiotic stress-tolerant plants is a desirable system but limited to those crop species for which protoplast-to-plant protocols have been developed (for review see Eeckhaut et al. 2013; Davey et al. 2005). The advantage of using protoplasts in *in vitro* selection systems is the opportunity to expose single cells at a physiologically uniform stage, in much larger populations compared to other plant source material, to a uniform action of the selective agent. Since a simple and efficient protocol for plant regeneration from immobilized carrot protoplasts has been available (Grzebelus et al. 2012) that technique seems to be the perfect tool to generate variation also under NaCl selection pressure. Additionally, the used accessions of Iranian carrot landraces were valuable source material for *in vitro* selection taking into account the high values of protoplast yield and the relatively high viability of the cells, similarly as was observed both in our model ‘Dolanka’ and other carrot accessions (Grzebelus et al. 2012).

The current study is one of the few reports presenting plant regeneration from single cells under high salinity (50–100 mM NaCl) in a long-term direct selection procedure, while most applications used callus or suspension cultures (Queiros et al. 2007; He et al. 2009; for a comprehensive review see; Rai et al. 2011). The toxic/lethal effect of selected doses of NaCl treatment was clearly demonstrated by cell death, the arrest of mitotic divisions in protoplast-derived cells or by interrupting developmental events during somatic embryogenesis in the carrot, finally leading to morphological abnormalities or tissue vitrification. Similar events such as cell death or decreasing embryogenic competence were also noted in sweet potato suspension cultures (He et al. 2009) or potato (Queiros et al. 2007) and wheat (Zair et al. 2003) callus cultures under saline pressure (20–150 mM). In contrast to developmental events during *in vitro* stages, it seems that NaCl treatment had no negative effect on plant acclimatization to *ex vitro* conditions, resulting in 90% efficiency in population of NaCl-treated plants in comparison to 93% for control. These results and our previous observations (Grzebelus et al. 2012) may suggest that this step strongly depends on proper environmental conditions applied during plant acclimatization, in particular, precise and gradual reduction of relative humidity.

Our study showed that 50 mM NaCl stress applied *in vitro* induced polyploidy of protoplast-derived regenerants in two out of three tested accessions. The increase in ploidy has been reported for regenerants subjected to biotic stress (Hartman et al. 1984; Rutkowska-Krause et al. 2003; Grzebelus et al. 2013); however to the best of our knowledge this is the first example of generating polyploidy under salt stress. Research on *Arabidopsis* showed, that plants with higher ploidy seem to be more adaptive to the adverse conditions compare to diploids what might be a result of

enrichment in genes related with response to different stress and hormonal responses observed under stress (del Pozo and Ramirez-Parra 2014, 2015).

In many studies salt stress was achieved by applying NaCl (Parihar et al. 2015; Piwowarczyk et al. 2016; Mozafari et al. 2018; Golkar and Taghizadeh 2018), while the soil solution of saline soils is composed of a range of dissolved salts (i.e. KCl, MgCl₂, MgSO₄, NaCl, Na₂SO₄, Na₂CO₃), each of which contributes to salinity stress. Therefore in this study in the greenhouse experiment, salt stress was the result of a mixture of different compounds delivered into the soil in the form of chlorides, nitrates and sulfates and was applied throughout 8 weeks. The survival of carrot plants depended on the NaCl-treatment *in vitro*, the soil salinity in the pot experiment and the genotype. Plant death was most frequently observed among the Dolanka regenerants originating from the control cultures and later grown in saline soil. The plants of this accession decayed most frequently during the salt stress period. In the NL landrace, irrespective of the NaCl-treatment *in vitro*, approximately half of the plants grown in highly saline soil were lost particularly during the post-stress period. However, the regenerants of both those accessions obtained from 50 to 100 mM NaCl-treated protoplast cultures had a higher survival rate in saline soil compared to the regenerants obtained from the control cultures. The DAL landrace was the least sensitive to the applied stress treatments. As a consequence, only for this accession we observed a relatively high number of regenerants originating from 100 mM NaCl-treated protoplasts and grown in saline soil. The majority of regenerants in this accession, irrespective of the NaCl treatment *in vitro*, survived the 8-week salt stress in the greenhouse experiment.

Salt tolerance mechanisms vary in different plants (Munns and Tester 2008), and such studies have not been published for the carrot so far. Plants subjected to elevated soil salinity may accumulate excessive amounts of ions (Na⁺, Cl⁻), primarily in the leaf blades. A failure to exclude Na⁺ from leaf blades manifests its toxic effect after days or weeks, depending on the species, and causes premature death of old leaves (Roy et al. 2014). In our study, dying of older leaves in plants was observed during the first weeks after planting into salinized soil and was coupled with a change of plant growth habit from erect to more prostrate, especially in the Dolanka and NL landrace. Additionally, in both accessions a change in leaf color, from green to yellow-green, was observed in plants grown in saline soil. The change in coloration might be explained by the degradation of photosynthetic pigments under salinity (Sultana et al. 2000; Sayyad-Amin et al. 2016).

The decline in leaf growth is considered one of the earliest responses to salt stress in glycophytes (Fricke and Peters 2002) and is primarily due to the osmotic effect of the salt around the roots (Munns and Tester 2008). The growth

rate measurement in our study was based on the length of leaves and petioles. In all accessions, leaf growth reduction was most significant at the highest salinity level, where the highest plant mortality was also observed. The carrot plants grown in moderately saline soil had a higher survival rate, and the leaf growth was less affected, compared to plants grown in highest salinity. Moreover the regenerants of Dolanka obtained from 50 mM NaCl-treated protoplast cultures and grown in saline soil had longer leaves compared to the regenerants obtained from the control cultures.

The morphological analysis performed in this study revealed that some traits, especially anthocyanin coloration in petioles and leaf and petiole hairiness, were particularly intense in plants subjected to salt stress. Anthocyanins are water soluble natural pigments belonging to the flavonoid group and exhibit diverse functions which include: attracting pollinators, repelling herbivores and parasites, but also a role in camouflaging and mimicry (Lev-Yadun and Gould 2008). In carrots, anthocyanins are naturally present and determine the purple root coloration (Simon 2000; Budahn et al. 2014). In our study, the Dolanka regenerants exhibited anthocyanin accumulation in petioles, but only after exposure to saline conditions. In both tested landraces, anthocyanins in petioles were present regardless of the applied treatments. They were, however, strongly intense in the plants which were planted into saline soil and which survived the stress period. These results support the hypothesis that anthocyanin synthesis is involved in defense systems to environmental stresses (Matus et al. 2010) and suggest that the increase in anthocyanins in the carrot might be a form of adaptation to salt stress. Stimulation of anthocyanin synthesis by increasing salinity was also observed in maize roots (Kaliamoorthy and Rao 1994), red cabbage seedlings (Eryılmaz 2006) or tomato fruit (Borghesi et al. 2011). Salt-tolerant genotypes of rice showed a significantly higher accumulation of proline and anthocyanin compared to salt-sensitive genotypes (Chutipaijit et al. 2011). Similar observations were made for the potato (Daneshmand et al. 2010) the Chilean strawberry (Garriga et al. 2014) and *Arabidopsis* (Lotkowska et al. 2015). The mechanisms responsible for production of anthocyanin under salt stress conditions are so far not well understood.

The other trait associated with the response of the carrot to salt stress was the leaf and petiole hairiness. Leaf hairiness is under genetic control; however, environmental conditions such as water supply and temperature might affect this trait. Leaf hair density is composed of two traits: the leaf surface area and the number of hairs per leaf. The leaf size is primarily controlled by growth environment, whereas leaf hair initiation is genetically based (Wilkens et al. 1996; Roy et al. 1999). When the environment influences the rates of cell division and/or cell expansion after the leaf hairs are differentiated, modifications of leaf hair density might

also occur (Pandey et al. 2016). This might be the explanation of what we observed in our study regarding the carrot. The DAL landrace regenerants from the control protoplast cultures had intermediate hairiness, while the plants regenerated from the 50 and 100 mM NaCl-treated protoplasts and planted into saline soil produced dense hairiness. Hairiness in this accession was observed not only on leaves and petioles, but also on inflorescence stems. In general, the Dolanka regenerants did not produce hairs on leaves and petioles, but some plants grown in highly saline soil had sparse and intermediate hairiness. It may be concluded that persisting salt stress affects the leaf size and plants produce shorter and smaller leaves covered with dense hairs. Small leaves with reduced surface area have also fewer stomata (Carins Murphy et al. 2014), which lowers the transpiration rate. Hairs on the leaves could reduce leaf convection and transpiration (and thus temperature) by affecting the leaf boundary layer resistance (Vaz Monteiro 2016). Combining all of the above, smaller hairy leaves tend to reduce water loss and this might be another mechanism of adaptation of carrot plants to saline environment.

Cultivated carrots have been bred for non-bolting and are usually biennial; however, local landraces as well as the wild relatives of the carrot might be winter annual, annual or biennial (Brooks and Feeny 2004; Budahn et al. 2014). Our results showed that increased soil salinity might affect the time of flowering of the carrot, as some plants of Dolanka and the Iranian landrace DAL grown for 8 weeks in saline soil produced inflorescence without vernalization. Molecular control of flowering time together with interaction with soil salinity was studied in *Arabidopsis*, where delayed flowering in salt-treated plants was observed. The results showed salt-induced degradation of the GIGANTEA (GI) protein which delayed the activity of the *Flowering Locus* (*FT*) and activated the expression of *Brother of Flowering Time Locus* (*BFT*) and further affected the transcription factor required for floral initiation (Kim et al. 2013; Ryu et al. 2014).

Our results showed that salt stress affected pollen viability in the carrot. In Dolanka and the NL landrace pollen viability decreased with increasing soil salinity, which also resulted in a reduced seed yield after self-pollination. However, the DAL regenerants derived from the 50 mM NaCl-treated cultures, irrespective of soil salinity, had highly (93–96%) viable pollen and produced a relatively high number of seeds. The inhibitory effect of salt stress on plant fertility may be due to differential competition in the supply of carbohydrates between vegetative growth and a constrained supply of these to the developing inflorescence (Parihar et al. 2015), as well as due to the excessive accumulation of sodium ions in flower organs. Khatun et al. (1995) found a correlation between the amount of sodium ions in pollen and pollen viability. Rice genotypes which accumulated more Na⁺ in their pollen produced less-viable

pollen grains. Moreover, the stigma receptivity was also affected in salt-treated plants, which, coupled with the reduced viability of pollen under stress condition, might be responsible for the failure of seed set (Khatun et al. 1995; Abdullah et al. 2001). The deleterious effects of salt stress resulting in seed yield reduction were reported for the durum wheat and barley (Munns et al. 2006), rice (Hasanuzzaman et al. 2009), rapeseed (Abbaszadeh et al. 2012), or beans (Nahar and Hasanuzzaman 2009).

Conclusions

It has been shown, for the first time that protoplast cultures can be used for enhancing salinity tolerance in carrot. The analysis of the obtained data suggests that salt stress applied to carrot protoplast cultures creates variation which allows alleviating the negative effects of salt stress on plant development and reproduction. The occurrence of the *de novo* variation under salt stress was also manifested by generation of polyploidy among obtained regenerants. The plants regenerated from the 50 and 100 mM NaCl-treated protoplast cultures developed functional mechanisms of salt tolerance and were able to survive and grow in soil with high salt concentrations as well as to produce seeds. The adaptation of the carrot to soil salinity is likely associated with accumulation of anthocyanins and increased hairiness. The increased level of anthocyanins might enhance antioxidant properties, while hairiness affects leaf convection and transpiration. The variation generated using the system presented in this paper may be used to enhance resistance to salinity but also potentially other abiotic stresses in the carrot.

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Author's contribution AK, EG designed the study; EG, KM performed protoplast cultures and plant regeneration; AK, AL-K performed the greenhouse experiment; EG performed data collection, analysis and statistics from in vitro experiment, drafted sections of the manuscript; AK concept, performed data collection, analysis and statistics from greenhouse experiment and prepared the initial and final version of the manuscript. All Authors read, reviewed and approved the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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