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RESEARCH ARTICLE

Mitigation by sodium nitroprusside of the effects of salinity on the morpho-physiological and biochemical characteristics of *Rubus idaeus* under in vitro conditions

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Abstract This study examined the changes brought about by sodium nitroprusside (SNP) in the effects of salinity on the morpho-physiological and biochemical characteristics of *Rubus idaeus* var. Danehdrosht. Raspberry shoot-tip explants were cultured on Murashige and Skoog medium supplemented with a growth regulator that combined benzyleadenine (1 mg/l), indol-3-butyric acetic acid (0.2 mg/l), SNP (0, 50 and 100 μM) and sodium chloride (0, 50 and 100 mM). The results showed that salinity stress significantly decreased morpho-physiological and biochemical characteristics such as RWC, MSI and total protein content in regenerated explants and significantly increased the total soluble sugar, proline contents, peroxidase and superoxide dismutase activity in compared to the control. However, SNP treatments mitigated the impacts of salinity on morphological and physiological characteristics in raspberry shoot-tip explants by increasing the accumulation of proline content, total protein content and total soluble sugar in line with increasing antioxidant enzyme activity under salinity conditions.

Keywords Nitric oxide · Raspberry · Salinity stress · In vitro condition

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Introduction

Salinity is one of the most limiting abiotic stresses on crop growth and production in arid and semi-arid regions. Based on the literature, salinity stress is more destructive to growth and yield than other abiotic stresses (Mahajan and Tuteja 2005). It has been reported that about 23% of the world's cultivated lands are saline, of which 37% is sodic (Khan and Duke 2001). Soil salinity reduces the osmotic potential of water in soil so that plants have less ability to take in water. Water loss in plant cells crucially limits cell division and cell elongation; in addition, it affects the metabolic activity both inside and outside the plant's cells. Salinity generally increases the absorption of Na^+ and Cl^- while reducing the absorption of essential organic nutrients, further destroying plants' ability to grow and thrive (Tester and Davenport 2003).

Plant tissue culture techniques using a wide variety of tissues and cells offer a practical and promising approach to sustainable development of plants, and have increasingly been used for plant regeneration as well as in vitro screening of plant germplasm for salinity resistance (Dasgupta et al. 2008). A number of studies have examined the effects of salinity on different plants such as grape (Alizadeh et al. 2010), tomato (Amini and Ehsanpour 2005), citrus (Ghaleb et al. 2010), pistachio (Chelli-Chaabouni et al. 2010), kiwifruit (Sotiropoulos and Dimassi 2004) and those of the prunus genus (Sotiropoulos et al. 2006) under in vitro conditions. Plants generally respond to their surrounding environment with resistance mechanisms or adaptations. Resistance mechanisms under in vitro conditions depend on the degree of stress, different stages of development and duration of development, in addition to the material and quality of laboratory glassware (Shao et al. 2008).

A review of the literature reveals a number of ways to improve plants' resistance biotic and abiotic stresses; these ways include transmission of resistance genes, using resistant varieties, screening genotypes suitable for each region and applying chemical compounds. In this regard nitric oxide (NO), a small gaseous molecule that can diffuse, is known to form endogenous components in many biological systems that play numerous physiological roles (Del Rio et al. 2004). The effect of this compound is related to its ability to chemically react with oxygen radicals, iron and proteins containing thiol (Wendehenne et al. 2001). Evidence indicates that NO is involved in several cell processes such as growth, development, metabolism, respiration, death, maturity and response to biotic and abiotic stresses. The protective as well as toxic effects of NO in plants is related to the concentration of molecules, synthesis, transfer and removal efficiency. NO is produced under salt stress and acts as a second messenger that induces PM H^+-ATPase expression (Zhao et al. 2004).

NO induces stress tolerance in plants by neutralizing the deleterious effects of oxidative stress in membrane and reducing Na^+ transport from root to shoots (Guo et al. 2010). According to Sarropoulou et al. (2014), the application of sodium nitroprusside (SNP) under in vitro culture improved the growth characteristics of cherry. The use of SNP and salicylic acid increased the resistance of *Pinus eldarica* to salinity and improved its growth characteristics (Zamani et al. 2014). Uchida et al. (2002) reported that hydrogen peroxide (H_2O_2) and NO are the important signaling molecules in rice for resistance to abiotic stress. By increasing the activity of antioxidant enzymes in *Lycopersicum esculentum*, NO causes resistance to salinity (Hayat et al. 2012a).

Raspberry and red raspberry are common terms for the *Rubus idaeus* plant in the Rosaceae family. Raspberry naturally grows in forests and woodlands throughout North America, Europe and Asia (Simpson et al. 2001). The objective of the present study was to investigate the effect of exogenous SNP treatment on some morpho-physiological and biochemical characteristics of *R. idaeus* var. Danehdrosht under salinity stress in in vitro conditions, and to determine some plausible effects of NO in this condition.

Materials and methods

Plant material

The shoot-tip explants of *Rubus idaus* var. Danehdrosht plants free from symptoms of disease or wounds were separated from the plants and transported to MS (Murashige and Skoog 1962) basal medium supplemented with 1 mg/l BA + 0.5 mg/l IBA + 0.3 mg/l GA_3. To determine the effect of SNP on the morpho-physiological and biochemical characteristics of raspberry under salinity stress in in vitro conditions, the shoot-tip explants of *R. idaeus* sub cultured on MS basal medium were supplemented with 0.8% (w/v) agar and 3%(w/v) sucrose and 1.0 mg/l BA + 0.5 mg/l IBA + NaCl (0, 50, 100 mM) and SNP at three concentrations) 0, 50 and 100 μM(.Three salinity levels—the control, 50 and 100 mM NaCl—and three SNP levels—the control, 50 and 100 μM—were applied as treatments. Each treatment was replicated six times, each time incorporating four sub-replications (or four explants). The pH of the medium was adjusted to 5.8 and autoclaved at 121.6 °C for 15 min, with the SNP used as the stock solution. Since SNP is very unstable in light and at high temperatures, the solution was prepared in the dark with distilled water and added to media after being autoclaved. When the temperature of the medium fell to 55–60 °C, before gel formation in the medium, it was sterilized with 0.2 μm filters. The culture jars were placed in a growth chamber at 25 ± 2 °C temperature under a photo period of 16 h light and 8 h dark with cool white fluorescent lights at 60% humidity.

Growth parameters

Morphological parameters including number of shoots and leaves, plant height, dry and fresh weight of shoots and the percent of viability were measured. Three to five regenerated plants were collected when they were 6 weeks old for the measurement of fresh and dry weight. Regenerated plants were weighed individually for their fresh weight and kept in black paper bags. Bags were then kept in an oven at 70 °C for 24 h, after which dry weight was determined by weighing the dried shoots. For calculation of dry matter (biomass), the following formula was used (Yin et al. 2005):

$$\text{Biomass}(\%) = [\text{dry weight (g)}/\text{fresh weight (g)}] \times 100.$$

Leaf relative water content (RWC)

The plant leaves in the jars were harvested and weighed (fresh weight), and then the leaves were placed in distilled water in a cool, dark place (4 °C) for 24 h until saturation, at which point the turgid leaves were weighed (turgid weight). The leaves were then dried in an oven at 70 °C for 24 h and weighed again (dry weight). The RWC of the leaves was evaluated according to method put forward by Galmés et al. (2007) using the following formula: RWC = [(fresh weight − dry weight)]/[(turgid weight − dry weight)] × 100.

Membrane stability index (MSI)

Leaf MSI was determined according to the method of Sairam et al. (2002). Leaf pieces (0.1 g) were cut and washed with double-distilled water. Thereafter, they were placed in 10 ml distilled water at 40 °C for 30 min. Then, electrical conductivity (C_1) was measured by an EC meter (RS 232 Conductivity meter, 8302). The samples were then placed in a boiling-water bath (100 °C) for 20 min and their electrical conductivity was recorded (C_2). The MSI was calculated based on the following formula: $MSI = [1 - (c_1/c_2)] \times 100$.

Leaf chlorophyll (chl.) and carotenoids (car.) content

Approximately 0.1 g of leaf was weighed for each plant sample and homogenized with liquid nitrogen. The sample was then ground, and 10 ml 80% acetone was added to extract the pigments. Next, the sample was centrifuged (HERMLE, Z 206 A, Germany) at 5000 rpm for 5 min and the supernatant was precisely separated and placed in a new tube. Finally, the absorbance of extracts was measured at 470, 663 and 645 nm using a spectrophotometer (UV-2100, New Jersey suv S2100). The contents of chl. a and b and car. were calculated by applying the method of Lichtenthaler and Buschmann (2001) on the basis of mg chl/g fresh weight.

$$\text{Chl. a} = (12.25\ A_{663.2} - 2.79\ A_{646.8})$$

$$\text{Chl. b} = (21.50\ A_{646.8} - 5.10\ A_{663.2})$$

$$\text{Chl. T} = \text{chl. a} + \text{chl. b}$$

$$\text{Car.} = [(1000\ A_{470} - 1.82\ \text{Chl. a} - 85.02\ \text{Chl. b})/198]$$

Analysis of total soluble sugar content

To measure the content of total soluble sugar, the anthrone reagent-based method was used (Irigoyen et al. 1992), in which 0.1 g of the sample was frozen using liquid nitrogen and ground, and 5 ml of 95% alcohol ethylic was added and homogenized. Then the supernatant was picked up and the residual leaf homogenized again with 10 ml of 70% ethyl alcohol. The mixture was centrifuged (HERMLE, Z 206 A, Germany) at 3500 rpm for 10 min. After centrifugation, 1 ml of the supernatant was separated and mixed gently with 3 ml of 70% anthrone reagent. The mixture was placed in a water bath at 100 °C for 10 min. Next, the reaction mixture was placed in an ice tank for 5 min. Total soluble sugar content was measured using a spectrophotometer (UV-2100, New Jersey suv S2100) at 625 nm. The total soluble sugar content was calculated using the glucose standard and expressed in mg/g fresh weight (mg/g FW).

Proline content

Proline content was calculated according to the Bates et al. (1973) method, in which 0.5 g of fresh leaf was frozen using liquid nitrogen and ground, then homogenized with 5 ml of 3% sulfosalicylic acid. The extracted compound was centrifuged (HERMLE, Z206A, Germany) at 6000 rpm for 5 min, and 2 ml nynhidrin and 2 ml of acetic acid were added to 2 ml of the supernatant. Then, the sample was placed in a boiling water bath (100 °C) for 1 h and was incubated in an ice tank for 5 min. Next, 2 ml of toluene was added to each test tube, which was quickly shaken with a shaker until two distinct phases formed. The upper phase was used for measuring absorbance with a spectrophotometer (UV-2100, New Jersey suv S2100) at 520 nm. The content of proline was calculated as milligram per g fresh weight (mg/g FW).

Total soluble protein

Total soluble protein was measured according to the Bradford (1976) method. Using this method, 0.5 g of fresh leaf was frozen using liquid nitrogen ground. During grinding 50 mg polyvinylpyrolidone (PVP) and 1.5 ml potassium phosphate buffer containing sodium meta bisulfite (0.01 g in 100 ml buffer) were added. The sample was homogenized and the extracted compound was centrifuged (Hi-tech, Micro, Germany) at 15,000 rpm and 4 °C for 20 min. Next, 175 µl of 50% glycerol was added to 500 µl of the supernatant. The mixture was divided between the test tubes and then placed in a freezer at −80 °C. Total soluble protein was measured using the Coomassie brilliant blue (G250) reagent. Then, 20 µl of the extracted mixture and 980 µl of Bradford reagent were mixed, and after 5 min, the amount of protein was measured spectrophotometrically (UV-2100, New Jersey suv S2100) at 595 nm. The albumin fraction (BSA) standard was used to calculate the total soluble protein content, which was expressed in mg/g fresh weight (mg/g FW).

Antioxidant enzyme activities assay

To assay antioxidant enzyme, 0.5 g of fresh leaf tissue was homogenized in 5 ml of 50 mM phosphate buffer (pH 7.0) containing 1% PVP. The homogenate was centrifuged at 15,000 rpm at 4 °C for 10 min and the supernatant was used as an enzyme source.

Peroxidase activity

POD activity was assayed by the procedure given by Hemeda and Klein (1990). To measure POD activity, 780 µl of the 50 mM potassium phosphate buffer (pH 7),

Table 1 Effect of sodium nitroprusside (SNP) on morphological characteristics of raspberry var. Danehdrosht under salinity stress in in vitro conditions

Treatment	Number of shoot	Number of leaf	Shoot height (cm)	Shoot Fresh weight (g per plant)	Shoot Dry weight (g per plant)	Biomass (%)
Control	3.91 ± 0.22c	6.58 ± 0.284cd	1.5 ± 0.109c	0.41 ± 0.006c	0.143 ± 0.0023c	65.12 ± 0.30c
SNP_1	8.5 ± 0.64a	13.83 ± 0.44a	3.67 ± 0.134a	0.619 ± 0.011a	0.167 ± 0.002a	73.02 ± 0.476a
SNP_2	6.5 ± 0.64b	11.58 ± 0.41b	2.32 ± 0.124b	0.518 ± 0.014b	0.159 ± 0.0026b	69.3 ± 0.21 b
$NaCl_1$	1.62 ± 0.23f	3.91 ± 0.284fg	1.025 ± 0.071de	0.219 ± 0.003f	0.119 ± 0.0007e	45.66 ± 0.75e
Na_1SNP_1	2.75 ± 0.32de	7.16 ± 0.284c	1.83 ± 0.073c	0.396 ± 0.006c	0.122 ± 0.0011de	69.19 ± 1.193b
Na_1SNP_2	3 ± 0.2cde	5.75 ± 0.184de	1.68 ± 0.093c	0.401 ± 0.006c	0.123 ± 0.0008de	69.32 ± 0.270b
$NaCl_2$	1.37 ± 0.23f	3.25 ± 0.142g	0.837 ± 0.058e	0.17 ± 0.003g	0.111 ± 0.0004f	34.70 ± 0.31f
Na_2SNP_1	2 ± 0.2ef	5 ± 0.18ef	1 ± 0.102e	0.359 ± 0.005d	0.129 ± 0.0011d	64.06 ± 0.329c
Na_2SNP_2	3.37 ± 0.23cd	5.7 ± 0.184de	1.375 ± 0.074 cd	0.297 ± 0.003e	0.124 ± 0.0018de	58.24 ± 0.44d
CV (%)	18.92	11.129	14.90	5.784	3.694	2.58

Mean ± standard error; means with the same letters are not significantely different

Control: 0 mM NaCl + 0 μM SNP, SNP_1: 50 μM SNP, SNP_2: 100 μM SNP, $NaCl_1$: 50 mM NaCl, Na_1SNP_1: 50 mM NaCl + 50 μM SNP, Na_1SNP_2: 50 mM NaCl + 100 μM SNP, $NaCl_2$: 100 mM NaCl, Na_2SNP_1: 100 mM NaCl + 50 μM SNP, Na_2SNP_2: 100 mM NaCl + 100 μM SNP

90 μl of the 1% glaichol and 90 μl of the 0.3% peroxide hydrogen (H_2O_2) were used. The reaction mixture was placed in an ice bed and poured into a covet. Next, 40 μl of the extracted enzyme was added, and the amount of POD was measured using a spectrophotometer (Analytik Jena, Specird 210) at 470 nm for 2 min. POD activity was reported as μM H_2O_2 degraded per minutes per mg protein.

Superoxide dismutase activity

SOD activity was assayed by measuring its ability to inhibit the photochemical reduction of nitrobluetetrazolium (NBT) using the method introduced by Beauchamp and Fridovich (1971). A reaction mixture containing 50 mM phosphate buffer (pH 7), 75 μM NBT, 13 mM methionine, 100 μl of the 12 μM riboflavin, 0.1 mM EDTA and 0–100 μl enzyme extract were mixed and placed under a 15 W fluorescent lamp for 15 min. After 15 min, the test tubes were covered using black bags and the light was switched off. The reaction was stopped by switching off the light. The amount of SOD was measured using a spectrophotometer (Analytik Jena, Specord 210) at 560 nm. The amount of enzyme that caused a 50% inhibition in the photochemical reduction of NBT was considered as one enzyme unit (expressed as changes in the absorption in mg protein per minute at fresh weight).

Data analysis

This experiment was carried out as a factorial experiment based on a completely randomized design (CRD). The data for all parameters was analyzed using the analysis of variance (ANOVA) procedure of SAS software ver. 9.3. Means were compared using Duncan's multiple range tests at the 0.05 probability level. To draw the graphs, Sigma Plot software was used.

Results

Morphological characteristics

Salinity stress significantly decreased such morphological characteristics as shoot number, leaf number, shoot height, fresh weight, dry weight and biomass. The highest values for number of shoots and leaves, shoot height, fresh weight, dry weight and biomass were obtained with 50 μM of the SNP treatment without any stress, and the lowest values were obtained with 100 mM of the NaCl. Furthermore, SNP significantly improved these values in the salinity treatments (Table 1; Fig. 1).

Pigment content

As shown in Table 2, salinity also significantly decreased the values for chlorophyll, carotenoid, RWC and MSI ($P \leq 0.05$). ANOVA analysis in line with mean comparison showed that in all treatments, the SNP was associated with significant differences in most of the characteristics. Under the saline-free treatment, SNP did not have a significant effect on RWC and MSI compared to the control, but adding SNP significantly increased these two traits under salinity conditions compared to the saline-free treatment. The highest amounts of chl. a, b and ab were obtained with 100 μM of SNP, while the highest amount of carotenoids was observed with 50 μM of SNP.

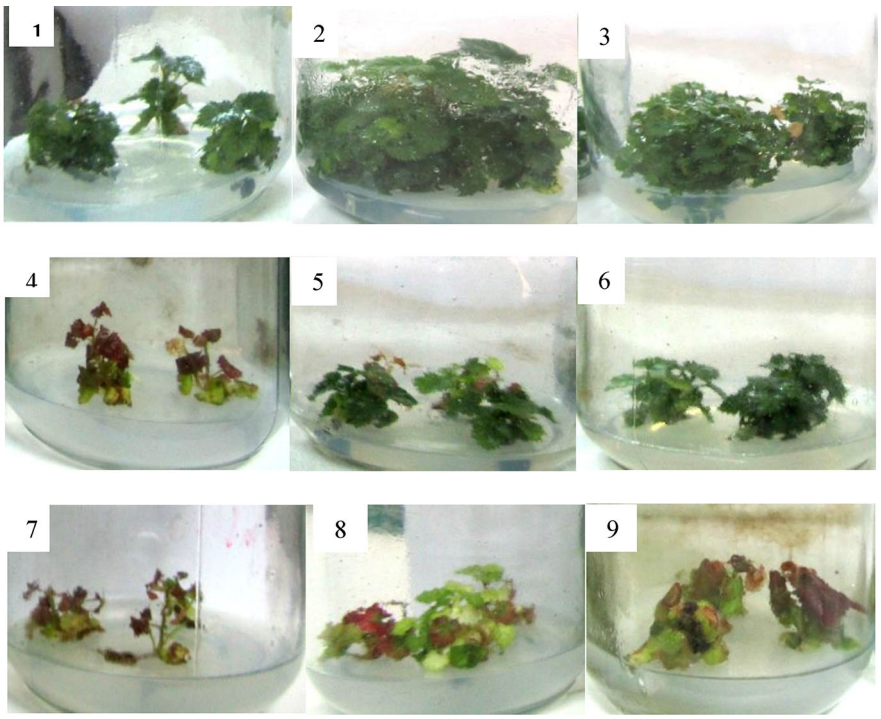

Fig. 1 Photos related to tissue culture of raspberry var. Danehdrosht affected by salinity stress and exogenous SNP; Treatments; *1* Control (0 mM NaCl + 0 μM SNP), *2* SNP_1 (50 μM SNP), *3* SNP_2 (100 μM SNP), *4* $NaCl_1$ (50 mM NaCl), *5* Na_1SNP_1 (50 mM NaCl + 50 μM SNP), *6* Na_1SNP_2 (50 mM NaCl + 100 μM SNP), *7* $NaCl_2$ (100 mM NaCl), *8* Na_2SNP_1 (100 mM NaCl + 50 μM SNP), *9* NA_2SNP_2 (100 mM NaCl + 100 μM SNP)

Table 2 Effect of sodium nitroprusside (SNP) on physiological characteristics of raspberry var. Danehdrosht under salinity stress in in vitro conditions

Treatment	Chl. a (mg g^{-1} FW)	Chl. b (mg g^{-1} FW)	Chl. ab (mg g^{-1} FW)	Car. (mg g^{-1} FW)	RWC (%)	MSI (%)
Control	0.753 ± 0.013c	0.35 ± 0.014c	1.104 ± 0.018c	0.25 ± 0.002c	82.13 ± 1.54a	84.59 ± 0.66a
SNP_1	0.927 ± 0.009b	0.502 ± 0.010b	1.430 ± 0.004b	0.314 ± 0.009a	86.57 ± 0.99a	87.53 ± 0.67a
SNP_2	0.985 ± 0.017a	0.636 ± 0.026a	1.621 ± 0.012a	0.267 ± 0.004b	85.82 ± 2.69a	84.81 ± 0.35a
$NaCl_1$	0.364 ± 0.010e	0.153 ± 0.015f	0.517 ± 0.008fg	0.178 ± 0.004e	59.76 ± 2b	66.37 ± 0.79c
Na_1SNP_1	0.464 ± 0.018d	0.227 ± 0.010def	0.692 ± 0.011e	0.211 ± 0.005d	78.15 ± 0.829a	76.68 ± 1.37b
Na_1SNP_2	0.513 ± 0.016d	0.288 ± 0.012cd	0.802 ± 0.006d	0.176 ± 0.004e	78.69 ± 1.39a	74.62 ± 0.82b
$NaCl_2$	0.225 ± 0.009f	0.160 ± 0.012ef	0.385 ± 0.019h	0.117 ± 0.004f	48.33 ± 2.95c	52.42 ± 1.64d
Na_2SNP_1	0.354 ± 0.015e	0.232 ± 0.011de	0.587 ± 0.015f	0.180 ± 0.001e	63.75 ± 2.30b	65 ± 1.91c
Na_2SNP_2	0.275 ± 0.009f	0.209 ± 0.004ef	0.484 ± 0.013g	0.165 ± 0.001e	77.22 ± 1.149a	56.51 ± 0.53d
CV (%)	7.10	13.91	4.66	6.72	10.637	6.009

Mean ± standard error; means with the same letters are not significantely different

Control: 0 mM NaCl + 0 μM SNP, SNP_1: 50 μM SNP, SNP_2: 100 μM SNP, $NaCl_1$: 50 mM NaCl, Na_1SNP_1: 50 mM NaCl + 50 μM SNP, Na_1SNP_2: 50 mM NaCl + 100 μM SNP, $NaCl_2$: 100 mM NaCl, Na_2SNP_1: 100 mM NaCl + 50 μM SNP, NA_2 SNP_2: 100 mM NaCl + 100 μM SNP. FW: fresh weight

Proline and total soluble sugar

Salinity stress significantly increased the proline and total soluble sugar (Fig. 2a, b). SNP significantly improved the proline and total soluble sugar contents. The highest amount of proline was observed with 100 μM SNP + 100 mM NaCl, and lowest amount of proline was observed in the control sample (Fig. 2a). The highest amount of total soluble sugar was observed with 100 mM NaCl without SNP, while the lowest amount of total soluble sugar was observed with 50 and 100 μM of SNP application without salinity (Fig. 2b).

Protein and antioxidant enzyme

Salinity significantly decreased the total soluble protein and increased the POD and SOD activity (Fig. 3a–c).The highest amount of total soluble protein was obtained with 50 μM SNP, and lowest amount of total soluble protein was observed with 100 mM NaCl. SNP increased the

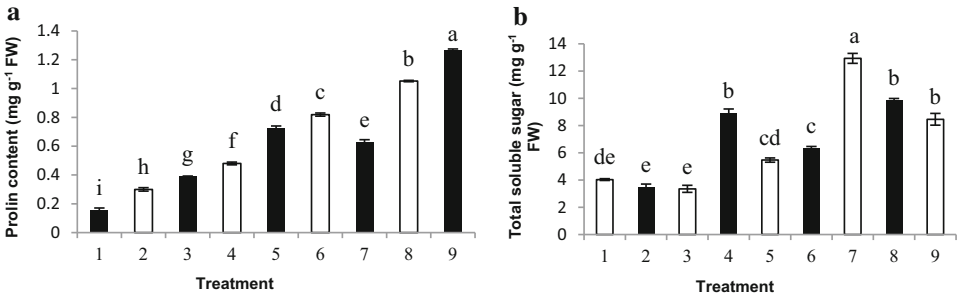

Fig. 2 Effect of sodium nitroprusside (SNP) on proline content (**a**), total soluble sugar (**b**) of raspberry var. Danehdrosht under salinity stress and in vitro conditions. Means of six replications ± standard error followed by *letters* indicating significant difference between mean using Duncan's methods ($P < 0.05$); means with the *same letters* are not significantly different. Treatments; *1* Control (0 mM NaCl + 0 μM SNP), *2* SNP_1 (50 μM SNP), *3* SNP_2 (100 μM SNP), *4* $NaCl_1$ (50 mM NaCl), *5* Na_1SNP_1 (50 mM NaCl + 50 μM SNP), *6* Na_1 SNP_2 (50 mM NaCl + 100 μM SNP), *7* $NaCl_2$ (100 mM NaCl), *8* Na_2SNP_1 (100 mM NaCl + 50 μM SNP), *9* NA_2SNP_2 (100 mM NaCl + 100 μM SNP). *FW* fresh weight

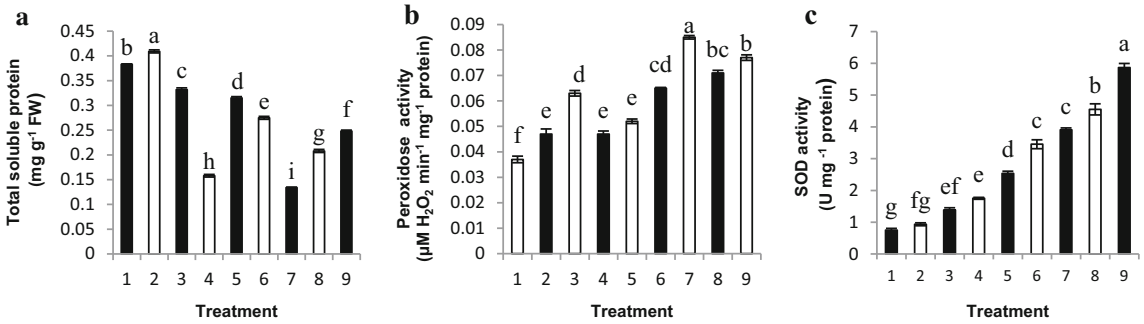

Fig. 3 Effect of sodium nitroprusside (SNP) on total protein content (**a**), POD activity (**b**), SOD activity (**c**) of raspberry var. Danehdrosht under salinity stress and in vitro conditions. Means of six replications ± standard error followed by *letters* indicating significant difference between mean using Duncan's methods ($P < 0.05$); means with the *same letters* are not significantly different. Treatments; *1* Control (0 mM NaCl + 0 μM SNP), *2* SNP_1 (50 μM SNP), *3* SNP_2 (100 μM SNP), *4* $NaCl_1$ (50 mM NaCl), *5* Na_1SNP_1 (50 mM NaCl + 50 μM SNP), *6* Na_1 SNP_2 (50 mM NaCl + 100 μM SNP), *7* $NaCl_2$ (100 mM NaCl), *8* Na_2SNP_1 (100 mM NaCl + 50 μM SNP), *9* NA_2SNP_2 (100 mM NaCl + 100 μM SNP). *FW* fresh weight

amount of total soluble protein both with and without the salinity treatment (Fig. 3a). Furthermore, salinity increased the POD and SOD activity in plants. The highest POD activity was observed with 100 mM NaCl, while the lowest POD activity was observed in the control. In contrast, SNP increased the activity of POD in both salinity and non-salinity conditions (Fig. 3b). Salinity stress along with SNP application significantly increased the SOD activity in plants The highest activity of this enzyme was measured with 100 mM NaCl + 100 μM SNP, and lowest one was observed in the control (Fig. 3c).

Percentage of viability

Salinity stress significantly decreased the percentage of viability of *Rubus* explants under in vitro conditions (Fig. 4). SNP treatment significantly increased the viability percentages of explants either with or without salinity stress. The survival rate decreased with increasing duration of salinity treatment. In all periods, the highest percentages of viability were achieved in the control samples for salinity with the application of both 50 and 100 μM SNP, while the lowest percentage of viability was observed with 100 mM NaCl at all durations (Fig. 4a–c).

Correlation between morpho-physiological and biochemical characteristics

The results of paired linear correlation presented in Table 3 showed that among vegetative characteristics, except biomass, there were positive correlations with MSI, RWC, protein and total chlorophyll at $P \leq 0.01$, and negative correlations with proline and SOD at $P \leq 0.05$ and $P \leq 0.01$. MSI had positive correlations with RWC, protein and total chlorophyll, and negative correlations with proline and SOD. RWC had a positive correlation with total chlorophyll and a negative correlation with proline. Proline was positively correlated with POD and SOD and negatively correlated with protein and total chlorophyll. Protein showed a positive correlation with total chlorophyll

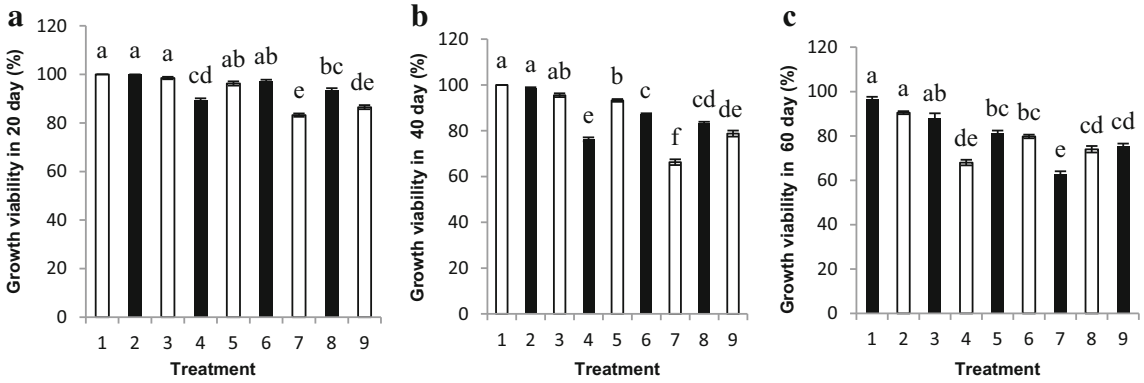

Fig. 4 Effect of salinity stress and time period on viability percent of explants in raspberry var. Danehdrosht under in vitro condition; **a** 20 days, **b** 40 days, **c** 60 days. Means of six replications ± standard error followed by *letters* indicating significant difference between mean using Duncan' methods ($P < 0.05$); means with the *same letters* are not significantly different. Treatments; *1* Control (0 mM NaCl + 0 μM SNP), *2* SNP_1 (50 μM SNP), *3* SNP_2 (100 μM SNP), *4* $NaCl_1$ (50 mM NaCl), *5* Na_1SNP_1 (50 mM NaCl + 50 μM SNP), *6* Na_1 SNP_2 (50 mM NaCl + 100 μM SNP), *7* $NaCl_2$ (100 mM NaCl), *8* Na_2SNP_1 (100 mM NaCl + 50 μM SNP), *9* NA_2SNP_2 (100 mM NaCl + 100 μM SNP)

Table 3 Pearson correlation between morpho-physiological and biochemical characteristics of raspberry var. Danehdrosht affected by salinity stress and SNP under in vitro conditions

Trait	Ln	Sh n	Bio	MSI	RWC	Pr	Sugar	Pro	Chl. II	POD	SOD
Ln	1	0.838**	0.148^{ns}	0.694**	0.550**	−0.401*	0.006^{ns}	0.518**	0.615**	-0.036^{ns}	−0.386*
Sh n		1	0.090^{ns}	0.689**	0.457**	−0.471**	0.001^{ns}	0.631**	0.661**	-0.221^{ns}	−0.479**
Bio			1	0.034^{ns}	0.118^{ns}	-0.147^{ns}	−0.788**	0.085^{ns}	0.083^{ns}	-0.029^{ns}	-0.107^{ns}
MSI				1	0.688**	−0.658**	0.092^{ns}	0.453**	0.540**	-0.275^{ns}	−0.626**
RWC					1	−0.335*	0.015^{ns}	0.193^{ns}	0.362*	-0.018^{ns}	-0.301^{ns}
Pr						1	0.066^{ns}	−0.587**	−0.522**	0.798**	0.976**
Sugar							1	0.005^{ns}	-0.146^{ns}	0.076^{ns}	0.037^{ns}
Pro								1	0.590**	−0.604**	−0.580**
Chl. II									1	−0.373*	−0.565**
POD										1	0.823**
SOD											1

Ln leaf number, *Sh n* shoot number, *Bio* biomass, *MSI* membrane stability, *RWC* relative water content, *Pr* proline, *Pro* protein, *Chl. II* total chlorophyll, *POD* peroxidase enzyme, *SOD* superoxide dismutase)

ns, *, ** non-significant, significant at $P \leq 0.05$ and $P \leq 0.01$, respectively

and negative correlations with POD and SOD. The total correlations of chlorophyll with POD and SOD were negative. Moreover, POD had a positive correlation with SOD (Table 3).

Discussion

Nitric oxide (NO) is a bioactive inorganic gaseous free radical, highly sensitive with low molecular weight and a half-life shorter than 6 s. This gas is known as a signaling and regulatory molecule in biological processes involved in regulating plant hormones such as cytokinin (Leshem and Kuiper 1996), auxin (Forde and Lorenzo 2001) and ethylene (Muday et al. 1995). NO is also involved in different cellular processes such as growth and development, metabolism, maturity, programmed cell death, respiration and response to various biotic and abiotic stresses. For example, NO in plants has been shown to alleviate the effects of drought stress (Lei et al. 2007a), osmotic stress (Zhao et al. 2009), heat stress (Ya'acov et al. 1998), salinity stress (Hayat et al. 2012b) and heavy-metal toxicity (Hsu and Kao 2004). It is stated that SNP is a releasing compound of NO in plants.

In the current study, salinity stress significantly decreased the examined vegetative characteristics and biomass; in contrast, the use of SNP under and free from salinity-stress conditions improved plant growth, which suggested that SNP might have a protective role in raspberry against salinity stress. Reduced plant growth under

saline conditions is probably attributed to the excessive absorption of sodium and chloride ions and ion toxicity (Kwon et al. 2005). Salinity reduces the water potential in soil, leading to a decline in cell turgidity pressure, which limits cells division and elongation. In contrast, reduction of water in the root zone increases abscisic acid (ABA) and decreases cytokinin production in the root, which in turn decreases cell division and plant growth (Tavallali et al. 2009; Lalinia et al. 2012). Based on our results, salinity reduced shoot growth and biomass, which is probably due to an increase in ABA synthesis and a reduction in cytokinin synthesis. Furthermore, this reduction in plant growth can be attributed to a reduction in RWC and MSI. Positive relationships between shoot and leaf number and biomass, on the one hand, and RWC and MSI, on the other, have been observed. The inverse relationship between biomass and salinity observed in the present study is in agreement with results reported by Zafar et al. (2015).

Our results showed that the use of SNP in salinity conditions improved the vegetative characteristics of raspberry. SNP under salt stress increased the activity of antioxidant enzymes, as well as levels of proline, carbohydrate, RWC and MSI, which led to improved plant growth. These results are similar to previous studies (Boon et al. 2013; Camilios-Neto et al. 2014). Improvement of plant growth under in vitro conditions might be due to the potential role of NO in the cytokinin signaling in the plant. NO has been shown to have an important role in stimulating the production of branches compared to branch elongation (Tan et al. 2001). Adventitious shoot regeneration from different explants have been shown to be affected by the concentration of SNP to varying degrees depending on their type (Kalra and Babbar 2010; Sarropoulou et al. 2014). High concentrations of SNP causes oxidative stress in the cell membrane, membrane degradation, increased ion transduction and ion imbalance, and ultimately decreases plant growth (Lai et al. 2010). Our results also demonstrated that SNP in high concentrations reduced growth, biomass and protein content while increasing anti-stress products such as proline and the activity of antioxidant enzymes.

Salinity stress significantly decreased chlorophyll a, b and ab, and carotenoids. Salinity causes metabolic disorders in plants such as a decrease in chloroplast activity and photosynthesis and increased respiration (Parida and Das 2005) followed by reduction in chlorophyll content (Blunden et al. 1996). The use of SNP increased the chlorophyll and carotenoid contents. SNP prevents intercostal chlorosis in leaves and increases the chlorophyll content. NO increases iron absorption by plants under stress conditions; iron playes an important role in the structure of chloroplast protein units (Ashraf and Bashir 2003).

Salinity stress reduced RWC and MSI in the current study. By increasing the osmotic potential of the nutrient solution in the root zone and reducing the water potential salinity caused a significant reduction in RWC percent. Reduced RWC under salinity stress has been also reported in pistachio (Panahi 2009) and pepper (Martınez-Ballesta et al. 2004). Our results showed that reduced RWC under salt stress coincided with a reduction in MSI to bring about a higher correlation between these two traits. The plasma membrane is the first part of the cytoplasm that encounters salinity, and the first area damaged by high salt. Salinity causes lipid peroxidation and damage to the cell membrane, thereby losing the membrane's selective-permeability characteristics and increasing electrolyte leakage (Kaya et al. 2006).

Exogenous SNP increased RWC and MSI under and free from salinity-stress conditions. SNP is a releasing compound of NO in the plants. NO increased MSI, antioxidant enzyme activity and accumulation of osmotic regulators such as proline and soluble sugar, and thereby maintained and increased plant RWC. NO acts as a free radical sweep and reacts with alcoxy lipid radicals and peroxyle lipids, directly stopping the lipid peroxidation chain. The role of NO in reducing membrane lipid peroxidation has previously been reported by Hsu and Kao (2004) and Laspina et al. (2005).

In the current study, salinity stress significantly increased the samples' proline content. In plant cells, proline acts as an osmotic balancer between the cytoplasm and vacuoles. Glutamate ligase enzyme activity increases in stress conditions, and glutamate is the precursor of chlorophyll and proline, so that more is spent to produce the proline content (Molazem et al. 2010). Exogenous SNP increases proline content under and free from salinity stress. In agreement with the present study, application of SNP has been shown to increase the levels of compatible solutes such as proline and glutathione (Tan et al. 2008). Lei et al. (2007b) demonstrated that NO induced and increased the activity of pyrolin-5-carboxylate synthetase (P5Cs) in the synthesis of proline in wheat under drought stress.

Salinity significantly increased the total soluble sugar, while SNP application reduced the total soluble sugar to a greater extent than the control sample under saline and non-saline conditions. Sugars from organic solutes involved in osmotic adjustment maintain cells' turgidity and sustain the stability of proteins and cell membranes (Ashraf and Harris 2004). Accumulation of soluble sugars such as sucrose, glucose and fructose are closely related to stress tolerance in plants. SNP application reduced the total soluble sugar compared to the control in salinity and non-salinity conditions. NO has been shown to increase the photosynthetic function and stimulate the metabolism of

carbohydrates. The reduction of soluble sugar content in tension through applying SNP can be due to SNP's ability to improve plant growth and soluble-sugar intake (Saed-Moucheshi et al. 2014b).

Salinity significantly decreased the total soluble protein content. Taking into consideration that proteins are the basic components of all cell activities, their reduction degrades enzymes and causes higher production of free radicals, as well as reducing protein synthesis (Saed-Moucheshi et al. 2014a). In contrast, SNP application increased soluble protein content; however, higher concentrations of SNP showed limited effect on the soluble protein content. SNP is a releasing compound of NO in plants, and acts as a signaling molecule to increase the activity of antioxidant enzymes such as SOD and CAT (Saed-Moucheshi et al. 2014a); this protects proteins and lipids against free radicals. High and significantly positive correlations between protein and both POD and SOD were also observed in this study. The increase of total soluble protein content by SNP application in peanuts (Verma et al. 2010) and in leaves of *Populus przewalskii* (Lei et al. 2007a) has also been reported.

In the current study, salinity significantly increased POD and SOD activity. Studies have suggested a link between stress resistance in plants and the activity of antioxidant enzymes (Hossain et al. 2015). SOD is cells' first line of defense against free radicals. POD combined with ascorbate peroxidase (APX) can neutralize hydrogen peroxide, converting it into water and oxygen in the plant cells. Increased POD enzyme activity under salinity stress has been reported for strawberry cultivars (Gulen et al. 2006).

SNP application under both salinity and non-salinity conditions increased POD and SOD activity. Verma et al. (2014) have demonstrated that SNP has a stimulatory effect on POD enzyme activity at low concentrations and an inhibitory effect at high concentrations. SNP releases NO, which itself increases the activity of the antioxidant enzymes' activities such as SOD activity, and converts superoxide ions to hydrogen peroxide and molecular oxygen; this protects cells against free radicals (Lei et al. 2007a). NO directly combines with superoxide anions to produce the peroxy nitrite radical ($ONOO^-$), which is less toxic, and thus causes relatively less damage, than the original superoxide anions (Beligni et al. 2002). In addition, NO reacts with lipid alcoxyl ($LO^{\cdot}$) and peroxyl ($LOO^{\cdot}$) to prevent the formation of lipid-degrading radicals (Lamotte et al. 2004).

In conclusion, this study found that salinity stress significantly decreased growth parameters, biomass accumulation, chlorophyll and carotenoid contents, RWC and MSI in *R. idaeus* explants. Free proline and soluble sugar were accumulated in order to osmotic adjustment and increase water absorption under salinity conditions. Salinity significantly decreased the total soluble protein and the percent of explants' viability, and increased the activity of enzymatic antioxidants including POD and SOD. Exogenous SNP application improves salinity resistance in plants by increasing the accumulation of proline and soluble sugars and activating antioxidant enzymes. The results of the current study show that salinity stress fostered the production of free radicals, which attacked macromolecules such as proteins and reduced plant growth. In contrast, SNP, by stimulating the enzymatic antioxidant system, increased the viability of plants under stress conditions and, specifically, improved plant resistance to salinity stress.

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