Molnar S *et al.* (2024)
Notulae Botanicae Horti Agrobotanici Cluj-Napoca
Volume 52, Issue 1, Article number 13691
DOI:10.15835/nbha52113691
Research Article

Investigation of salinity tolerance to different cultivars of highbush blueberry (*Vaccinium corymbosum* L.) grown *in vitro*

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Abstract

Salinity is one of the most critical abiotic stresses affecting various physiological, biochemical, and molecular functions of plants. This study aimed to assess the effects of different salt concentrations on *in vitro* blueberry shoots ('Bluecrop', 'Blueray', 'Brigitta Blue', 'Duke', 'Goldtraube', 'Hortblue Petite', and 'Patriot' cultivars) and to understand the mechanisms employed by this species under saline conditions. The Woody Plant Medium (WPM) proliferation medium was supplemented with 10, 50, 100, and 150 mM NaCl to induce salt stress. After ten weeks of *in vitro* culture under salinity stress, various parameters were evaluated, including the number of shoots obtained/explant, shoot length, fresh weight, dry weight, water content, stress tolerance index (STI), and McKinney index (MKI). Additionally, the behaviour of blueberry cultivars under salt stress was analysed using electron paramagnetic resonance spectroscopy (EPR). Compared to the control (culture medium without NaCl), all treatments with NaCl reduced shoot length and the number of shoots obtained/explant in all studied blueberry cultivars. 'Brigitta Blue' reported the shortest shoots (0.04 ± 0.02 cm) at a concentration of 150 mM NaCl, followed by 'Blueray' with 0.05 ± 0.03 cm. Also, the lowest number of shoots/explant was recorded for both cultivars under 150 mM NaCl, namely 0.12 ± 0.07 shoots/explant ('Brigitta Blue') and 0.11 ± 0.04 shoots/explant ('Blueray'). Salt tolerance, as expressed by ITS and MKI, confirmed that 'Goldtraube' exhibited higher salt tolerance, with the highest ITS values and the lowest MKI values. Further validation presented 'Goldtraube' as the most unresolved spectra of the Mn (II) hyperfine structure under all salt concentrations, therefore 'Goldtraube' was the most tolerant to saline stress.

Keywords: electron paramagnetic resonance spectroscopy; morphological parameters; Mn (II); salt stress; stress tolerance index; water content

Introduction

Soil salinity is one of the most important global problems that negatively affect crop productivity through water stress, excessive absorption of ions such as sodium (Na^+) and chloride (Cl^-), and nutritional

Received: 13 Feb 2024. Received in revised form: 20 Mar 2024. Accepted: 28 Mar 2024. Published online: 29 Mar 2024.
From Volume 49, Issue 1, 2021, Notulae Botanicae Horti Agrobotanici Cluj-Napoca journal uses article numbers in place of the traditional method of continuous pagination through the volume. The journal will continue to appear quarterly, as before, with four annual numbers.

imbalance (Isayenkov, 2012; Isayenkov and Maathuis, 2019). At present, out of 1.5 billion hectares of cultivated land around the world, according to FAO about 90 million hectares (6%) is affected by excess salt content (Parihar *et al*., 2015). Food production suffers when agricultural land becomes more salinized. Increasing crop plants tolerance to salinity stress is essential for boosting yield in environments with scarce water resources and high salinity (Zayed *et al*., 2017).

The gains obtained from blueberry cultivation are huge as the fruit price has been quite constant and remained high over the years (Wróblewska and Czernyszewicz, 2017). Like most perennial fruit crops, blueberry has a low salt tolerance. The decrease of peat bogs all over Europe (Chambers *et al*., 2013) and the increased interest in the growing of highbush blueberry drives growers to farm on less suited environments for this species (Wróblewska and Czernyszewicz, 2017). One of the primary obstacles preventing the development of new highbush blueberry plantations is the limited accessibility of suitable soils for cultivation (Ochmian *et al*., 2019).

Salinity inhibited the growth of highbush blueberries, however, the reaction varied according to the salinity's source (Bryla *et al*., 2021). Soil suitability for blueberry production is influenced by soil pH and EC, which are recognized as markers of acidity and salt concentration, respectively (Messiga *et al*., 2018).

Because highbush blueberries are extremely sensitive to soil salt increases, fertilizers that maintain the specific conductivity (EC) at <2 mS/cm ought to be applied in its cultivation (Machado *et al*., 2012). The highest dose of phosphogypsum raised the concentration of salt in the soil to the greatest extent, rendering it inappropriate for soil acidification in highbush blueberry farming. Reduced effects on the concentration of salt were observed for sulfur, urea phosphate, and sulfuric acid, at the lowest concentration. The most vulnerable material to salinity was loamy sand, and the least susceptible material was peat (Ochmian *et al*., 2021). Some granular fertilizers of N also contain sulfate, which can cause salt buildup and be detrimental to blueberry plants (Messiga *et al*., 2018).

Previous studies show that one of the most important criteria influencing the growth of highbush blueberry (*V. corymbosum* L.) is the pH of the soil, which must be maintained at low values (3.8-5.5), throughout the culture (Ortiz-Delvasto *et al*., 2023; Gallegos-Cedillo *et al*., 2018; Imler *et al*., 2019; Ochmian *et al*., 2021; Zhou *et al*., 2022). Generally, peat soils are most suitable for highbush blueberry but, currently, there is a decrease in these soils (Ochmian *et al*., 2019; Schreiber and Nunez, 2021). For acidification and enrichment of the substrate in organic matter, different methods can be use (Kozos and Ochmian, 2016). After many years of use they may adversely affect soils. For example, the use of acidifying fertilizers causes an increase in salinity (Ochmian *et al*., 2021) and soil salinity is becoming an increasing problem for production of highbush blueberry (Bryla *et al*., 2021; Muralitharan *et al*., 1992).

Assessment of salinity tolerance in the field can be constrained by season, affected by climate, or unreliable due to combined salinity and water stress problems, so more reliable and time-saving selection techniques have been developed using tissue culture technology (Khenifi *et al*., 2011). *In vitro* determination of salinity tolerance and morphological changes occurring under salt stresses were studied for different species grown *in vitro*, as follows: *Lycopersicon esculentum* (Amini *et al*., 2007), *Crithmum maritimum* (Grigoriadou and Maloupa, 2008), *Citrus macrophylla* (Pérez-Tornero *et al*., 2009), *Solanum* sp. (Khenifi *et al*., 2011; Zaki and Yokoi, 2016; Molnár *et al*., 2021; Zaki and Radwan, 2022a; Ortega-Albero *et al*., 2023), *Saccharum* spp. (Passamani *et al*., 2017), *Stevia rebaudiana* (Javed and Gürel, 2019), *Spinacia oleracea* (Muchate *et al.,* 2019), *Triticum turgidum* (Ami *et al.,* 2020), *Antigonon leptopus* (El-Zaiat *et al*., 2020), *Olea europaea* (Bashir *et al*., 2021), *Pfaffia glomerata* (Fortini *et al., 2023*), *Cicer arietinum* (Aasim *et al*., 2023), *Ficus carica* (Granata *et al*., 2023).

It states that the applications of electron paramagnetic resonance (EPR) spectroscopy in plant research have enabled the characterization of differences between sensitive and tolerant genotypes when exposed to various water or salt stress conditions in different plant species (Labanowska *et al*., 2013; Steffen-Heins and

Steffens, 2015; Filek *et al*., 2016). Furthermore, EPR spectroscopy enables the investigation of plant material without the need for extensive biochemical preparation of samples. While EPR is primarily applicable to systems containing unpaired electrons, it can still be utilized to examine biological tissues due to the presence of various paramagnetic centers. These include transition metal ions such as manganese (Mn), iron (Fe), and copper (Cu), which are found in enzymes and other biological structures.

The aim of the current study was to evaluate the degrees of salinity tolerance among different highbush blueberry cultivars using tissue culture techniques. Hence, the number of shoots obtained/explant, shoots length, fresh weight, dry weight, and water content were determined for seven highbush blueberry cultivars in the absence and presence of NaCl, during *in vitro* multiplication stage and the following salinity tolerance indices were calculated: stress tolerance index (STI), McKinney index (MKI). In addition, an attempt was made to elucidate the behaviour of the seven blueberry cultivars under salt stress using electron paramagnetic resonance (EPR) spectroscopy. The results represent a contribution to the understanding of the mechanisms adopted by highbush blueberry grown under salinity conditions.

Materials and Methods

In vitro saline stress

The explants used for this experiment were excised from the *in vitro* culture of highbush blueberry (12 weeks old) which had been cultured on Woody Plant Medium (WPM) (Lloyd and McCown, 1980) with 100 mg/L Sequestrene 138, 1 mg/L zeatin (Z), 3% (w/v) sugar, 0.4% (w/v) Plant agar, pH 5, according to the protocol of Clapa *et al*. (2018). Seven highbush blueberry (*V. corymbosum* L.) cultivars were used: 'Bluecrop', 'Blueray', 'Brigitta Blue', 'Duke', 'Goldtraube', 'Hortblue Petite', and 'Patriot'.

The culture vessels used were 720 ml glass jars, with a diameter of 9 cm and a height of 13.5 cm with sponge-vented screw caps. In each vessel, 100 mL of WPM proliferation medium, supplemented with four different NaCl concentrations (10, 50, 100, and 150 mM), was dispensed for culturing. All components, including NaCl, were added prior to pH correction and autoclaving at 121 °C for 20 min. The components were purchased from Duchefa Biochemie BV (Haarlem, Netherlands).

In each treatment, three jars were used and in each jar were inoculated 10 fragments of shoots without apical bud, each one of 1.5-2 cm. The *in vitro* cultures were incubated in the growth room for a 16-h photoperiod with 32.4 $\mu mol \cdot m^{-2} \cdot s^{-1}$ light intensity (Cool white fluorescent lamps, 36 W; Philips, Amsterdam, The Netherlands) at 22 ± 1 °C and 50% ± 2% humidity.

The data were collected after 10 weeks of culture for analysis. The analyzed data refer to the number of shoots obtained/explant (SN), the average length of shoots (SL), the fresh weight/explant (FW), dry weight (DW), and water content (WC). Fresh leaves obtained *in vitro* were used for the electron paramagnetic resonance (EPR) measurements.

Calculation of tolerance indices

Stress tolerance index (STI). Response to salinity stress for each blueberry cultivar was assessed using the stress tolerance index (STI). STI was calculated as the ratio of the trait performance at 10, 50, 100, and 150 mM NaCl to the trait performance at 0 mM NaCl according to the following formula (Zaki and Radwan, 2022b):

$$STI = Ts/Tp \quad (1)$$

where:

Ts is the trait of genotype under stress treatments.

Tp the trait of genotype under normal conditions.

The genotypes with high STI values will be tolerant to salinity stress.

McKinney index (MKI). Evaluation of the chloroses and/or necroses induced by salinity was performed by ranking each shoot into six classes (Table 1) using a modified McKinney Index (MKI) (Urbinati *et al.*, 2020) according to the following formula:

$$MKI = \Sigma(ni \times i)/N \quad (2)$$

Where:

– ni is the number of shoots assigned to the class,

– i is the numeric value of the class,

– N is the total number of examined shoots at each salt concentration.

Data are the mean value of 15 plants from three jars for each treatment except 150 mM NaCl where there were not enough shoots.

Table 1. Rating scale comprising six categories with corresponding numerical values assigned to the McKinney index (MKI)

Class	Symptoms
0	No injury
1	Partial chlorosis of leaves
2	Chlorosis of the basal leaves
3	Necrosis of the basal portion (10%) of the stem
4	60% of shoot necrosis
5	80% of shoot necrosis
6	100% of shoot necrosis

Electron paramagnetic resonance (EPR) spectroscopy

EPR spectra were measured with an X-band Bruker Elexsys 580 spectrometer. The spectra were recorded in a continuous wave at room temperature (22 ± 1 °C) with the following parameters: microwave power 9 mW, modulation amplitude 1 G, center field 3515 G, and scan field 50 G. The samples (fresh leaves) were macerated.

Statistical analysis

The experimental design was a 7×5 (cultivars × NaCl concentrations) factorial experiment with three replications. One-way ANOVA was performed for treatments within one cultivar and for all the cultivars within one treatment to investigate whether the differences in physiological parameters of the *in vitro* plants were affected by the presence of different concentrations of NaCl added to the culture media. Post hoc testing for the ANOVAs was performed using Tukey's honestly significant difference test (Tukey's test) using a $P<0.05$ significance level to determine the statistically significant differences between the means. Values shown (in text and figures) are means ± SE (standard error).

Results

Effect of NaCl on in vitro growth parameters

The general appearance of *in vitro* proliferated shoots was affected by the presence of all concentrations of NaCl in the culture media, and shoots with shorter inter-nodes and smaller and yellowed leaves were observed, as shown in Figure 1.

The length of the shoots was significantly influenced by the concentration of NaCl in the growth medium for all studied cultivars (Figure 2). The general trend was a decrease in shoot length with increasing NaCl content.

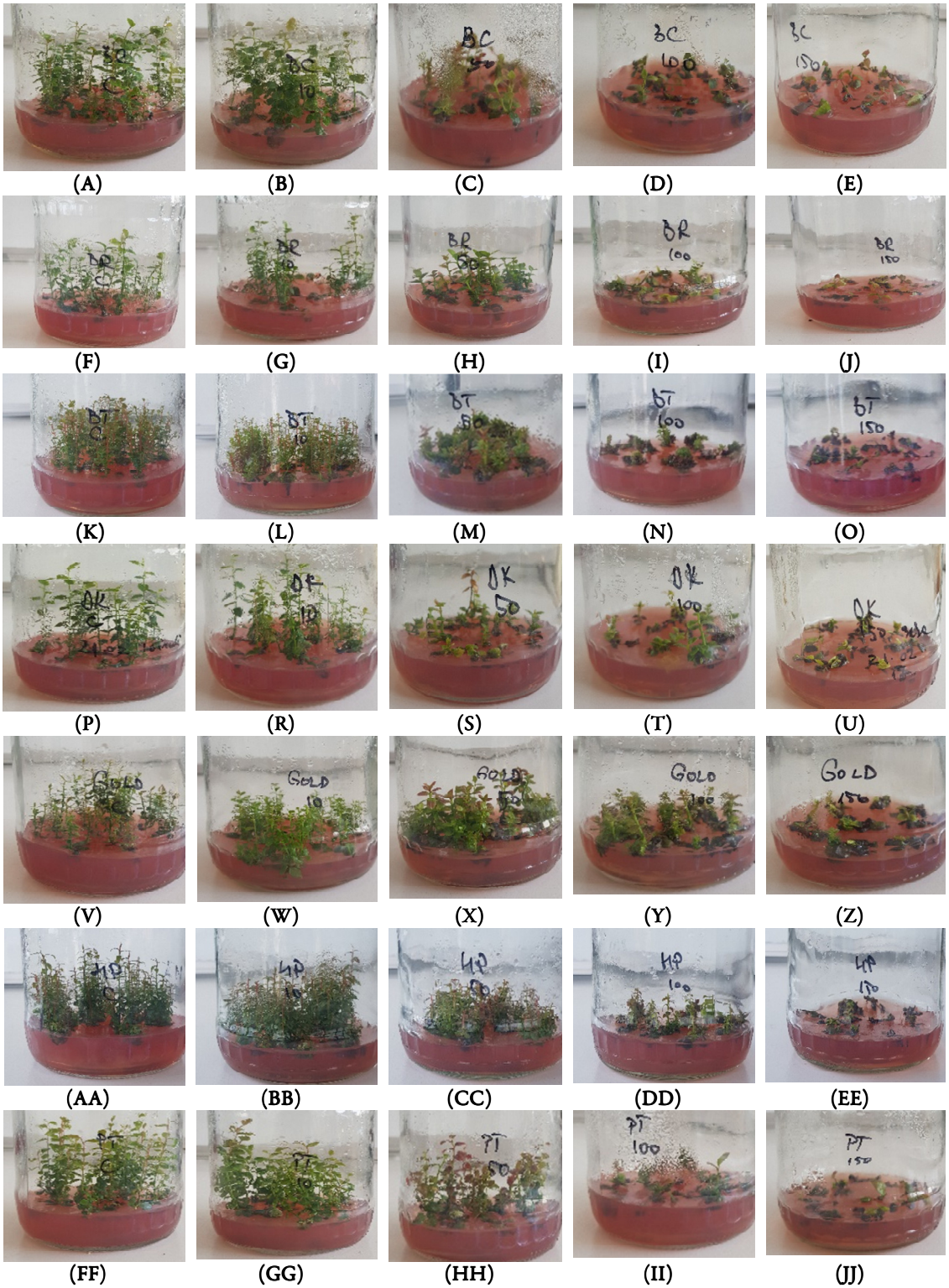
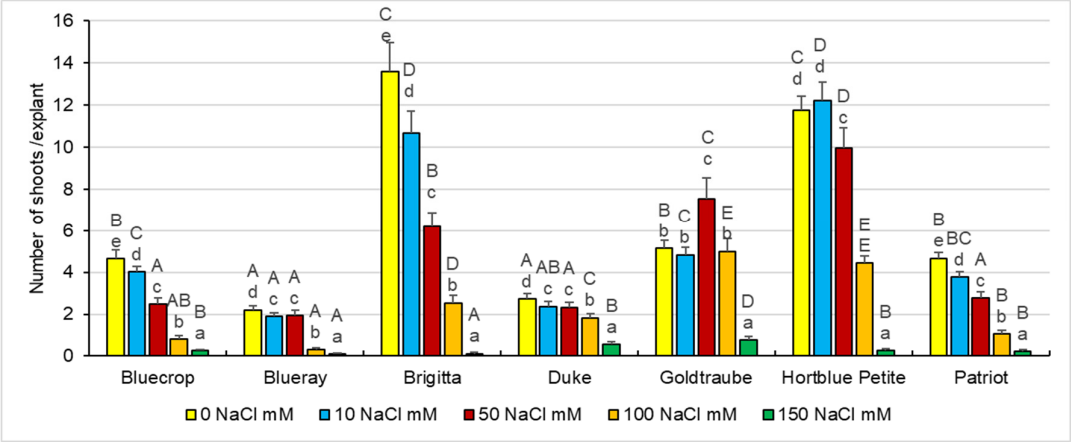


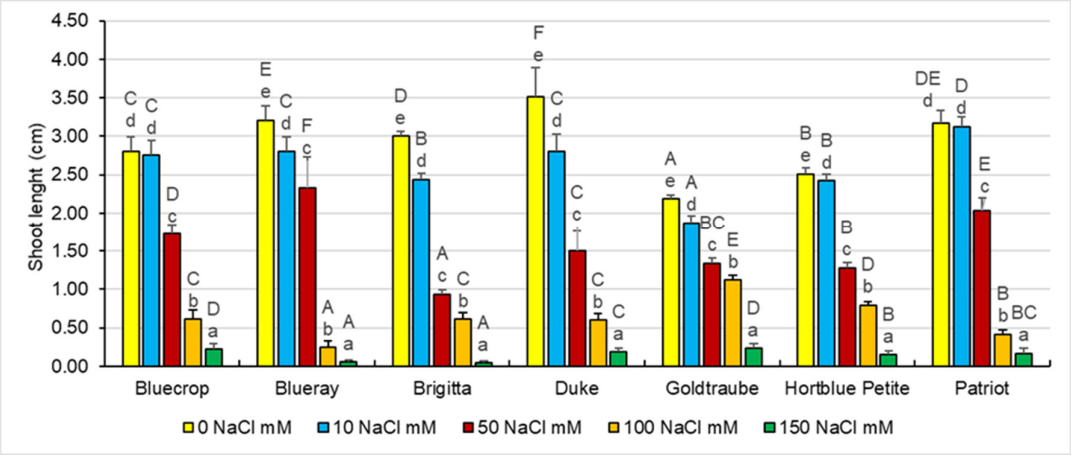
Figure 1. Effect of different concentrations of NaCl on *in vitro* culture of blueberry cultivars: 'Bluecrop' (A–E), 'Blueray' (F-J), 'Brigitta Blue' (K-O), 'Duke' (P-U), 'Goldtraube' (V-Z), 'Hortblue Petite' (AA-EE), 'Patriot' (FF- JJ) at 0, 10, 50, 100 and 150 mM NaCl (left to right)

All studied cultivars were found to have the longest shoots when cultivated in a NaCl-free culture medium, with lengths ranging from 3.52 ± 0.38 cm ('Duke') to 2.18 ± 0.05 cm ('Goldtraube'). The shoot length decreased with increasing NaCl concentration. 'Brigitta Blue' reported the short-est shoots (0.04 ± 0.02 cm) at a concentration of 150 mM NaCl, followed by 'Blueray' with 0.05 ± 0.03 cm (Figure 2A).

The same trend was observed regarding the number of shoots/explant, which decreased with increasing NaCl concentration. In terms of this parameter as well, the lowest number of shoots/explant were recorded for 'Brigitta Blue' (0.12 ± 0.07) and 'Blueray' (0.11 ± 0.04), as shown in Figure 2B.



(A)



(B)

Figure 2. Number of shoots/explant (A) and shoot length (cm) (B) of highbush blueberry cultivars. For inducing the salinity stress in culture media were added 0, 10, 50, 100, and 150 mM NaCl. Error bars indicate mean ± SE and different lowercase letters within each cultivar indicate significant differences among the treatments and different capital letters indicate significant differences among the cultivars undergoing the same treatment according to Tukey's HSD test ($P \leq 0.05$)

After removing the culture medium residues, the explants were weighed to determine their fresh weight. Concerning this parameter, a tendency towards decreased fresh weight was observed in the presence of 100 and 150 mM NaCl concentrations, particularly (as shown in Figure 3A). The lowest fresh weight was observed at the concentration of 150 mM NaCl for all the tested cultivars, with values ranging from 10.00 ± 1.35 mg ('Blueray') to 57.32 ± 7.04 mg ('Goldtraube'), as depicted in Figure 3A.

After drying the shoots for three days at 45 °C, the total water content was measured. As shown in Figure 3B, the total water content was similar to the control for most varieties when grown in media with 10 mM

NaCl or showed an increase under 50 and 100 mM NaCl. However, in the presence of 150 mM NaCl, the water content decreased compared to the control (without salt). Thus, the lowest water content was recorded in shoots derived from the culture medium supplemented with 150 mM NaCl and ranged from 50.67 ± 0.89% ('Brigitta Blue') to 63.57 ± 1.10% ('Goldtraube'). Blueberry shoots grown under 100 mM NaCl had the highest water content, with values ranging from 77.70 ± 0.61% ('Patriot') to 86.03 ± 0.69% ('Brigitta Blue'), as shown in Figure 3B.

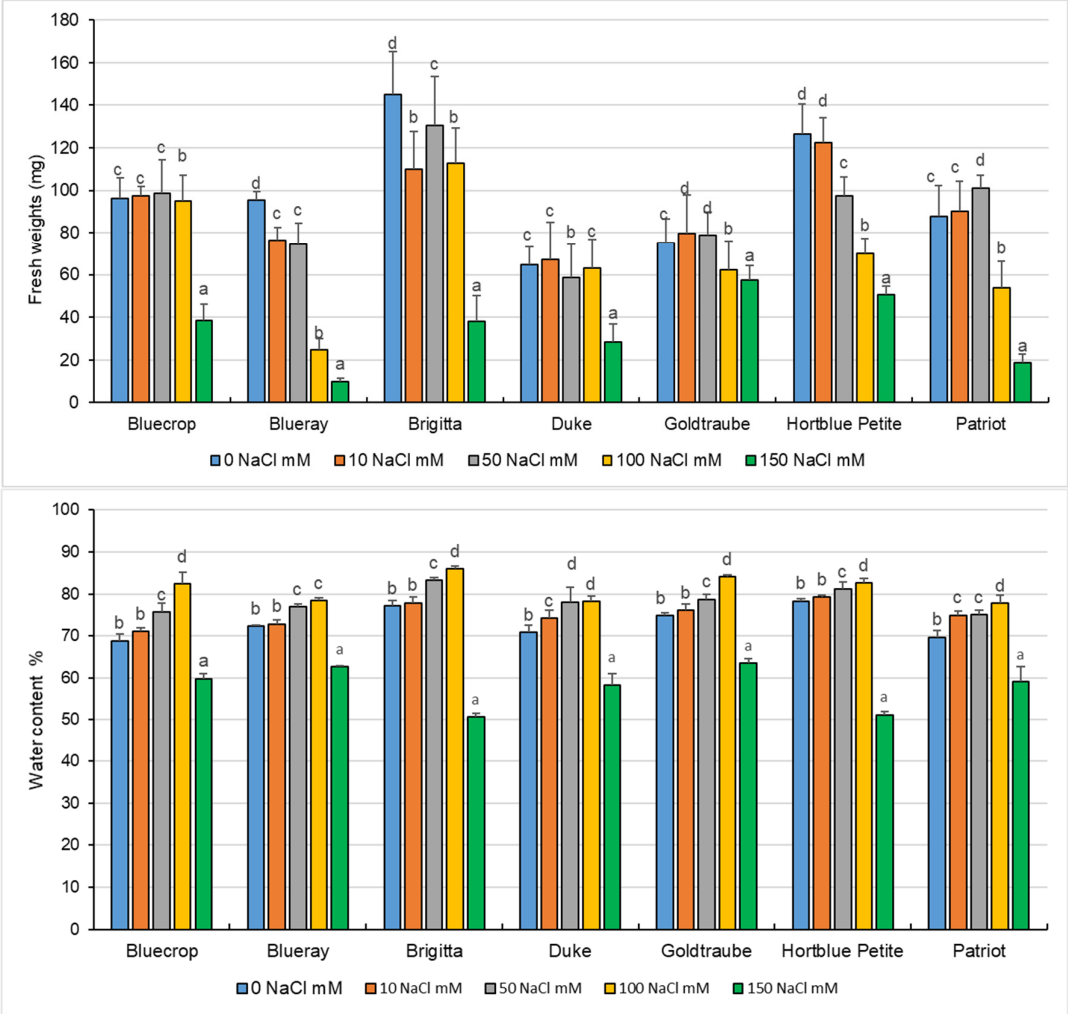


Figure 3. Effects of salinity stress on (A) fresh weight (FW mg), (B) water content (WC%) of seven *in vitro*-grown highbush blueberry cultivars. For inducing the salinity stress in culture media were added 0, 10, 50, 100, and 150 mM NaCl. Error bars indicate mean ± SE and different lowercase letters within each cultivar indicate significant differences among the treatments and different capital letters indicate significant differences among the cultivars undergoing the same treatment according to Tukey's HSD test ($P \leq 0.05$)

Tolerance indices

Salt tolerance, as expressed by the salt tolerance index (STI), is presented in Table 2. The general trend observed was a decrease in STI with increasing salinity levels. However, variations in STI were observed among different cultivars, ranging from low to high salinity tolerance. 'Goldtraube' exhibited the highest STI values

under high salt concentrations, indicating greater tolerance. As shown in Table 2, the lowest STI values were recorded for 'Brigitta Blue' under all salt concentrations and for all parameters.

Table 2. Stress tolerance index (STI) for seven blueberry cultivars maintained for ten weeks in culture media with different concentrations of NaCl

Trait	Cultivar						
	'Bluecrop'	'Blueray'	'Brigitta Blue'	'Duke'	'Goldtraube'	'Hortblue Petite'	'Patriot'
10 NaCl mM							
Number of shoots	0.87	1.83	0.30	1.48	0.79	0.34	0.86
Average length of shoots	0.99	0.87	0.81	0.80	0.86	0.96	0.99
Fresh weights (FW) (mg)	1.02	0.80	0.76	1.04	1.06	0.97	1.03
Dry weights (DW) mg	1.07	0.79	0.73	0.94	1.14	0.93	0.84
50 NaCl mM							
Number of shoots	0.53	0.88	0.46	0.85	1.47	0.85	0.59
Average length of shoots	0.61	0.73	0.31	0.43	0.61	0.51	0.64
Fresh weights (FW) (mg)	1.03	0.78	0.90	0.91	1.05	0.77	1.15
Dry weights (DW) mg	0.90	0.71	0.69	0.63	1.34	0.61	0.94
100 NaCl mM							
Number of shoots	0.17	0.14	0.19	0.67	0.97	0.38	0.23
Average length of shoots	0.22	0.08	0.20	0.17	0.51	0.32	0.13
Fresh weights (FW) (mg)	0.99	0.26	0.78	0.98	0.83	0.55	0.61
Dry weights (DW) mg	0.60	0.22	0.49	0.72	0.80	0.42	0.42
150 NaCl mM							
Number of shoots	0.05	0.05	0.01	0.20	0.15	0.02	0.05
Average length of shoots	0.08	0.02	0.01	0.05	0.11	0.06	0.05
Fresh weights (FW) (mg)	0.40	0.11	0.26	0.44	0.76	0.40	0.21
Dry weights (DW) mg	0.29	0.08	0.20	0.28	0.59	0.34	0.23

The McKinney index (MKI) was employed to assess the degree of shoot chlorosis and necrosis induced by NaCl treatment. As indicated in Table 3, MKI exhibited a positive correlation with increasing NaCl concentrations across all studied cultivars. In samples exposed to 10 mM NaCl, no chlorotic leaves or brown shoots were observed, and the MKI value was 0. For samples exposed to 50 mM NaCl, MKI values ranged from 1.27 ('Goldtraube') to 4.27 ('Brigitta Blue'). At 100 mM NaCl, 'Goldtraube' exhibited the lowest MKI value (3.20), while the highest MKI value was recorded in 'Brigitta Blue' (5.80).

Table 3. McKinney Index (MKI) for seven blueberry cultivars maintained for ten weeks in culture media with different concentrations of NaCl

NaCl (mM)	Cultivar						
	'Bluecrop'	'Bluray'	'Brigitta Blue'	'Duke'	'Goldtraube'	'Hortblue Petite'	'Patriot'
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00
10	0.00	0.00	0.00	0.00	0.00	0.00	0.00
50	2.20	1.93	4.27	3.13	1.27	1.87	1.73
100	5.67	5.60	5.80	5.27	3.20	4.73	5.13

Electron paramagnetic resonance (EPR) spectroscopy

The seven experimental plots analyzed show signals near of a centered value at 3500 G and are extended side by side from the center point, with 300 G (Figure 4). The g factor value corresponding to the centerline is 2.02.

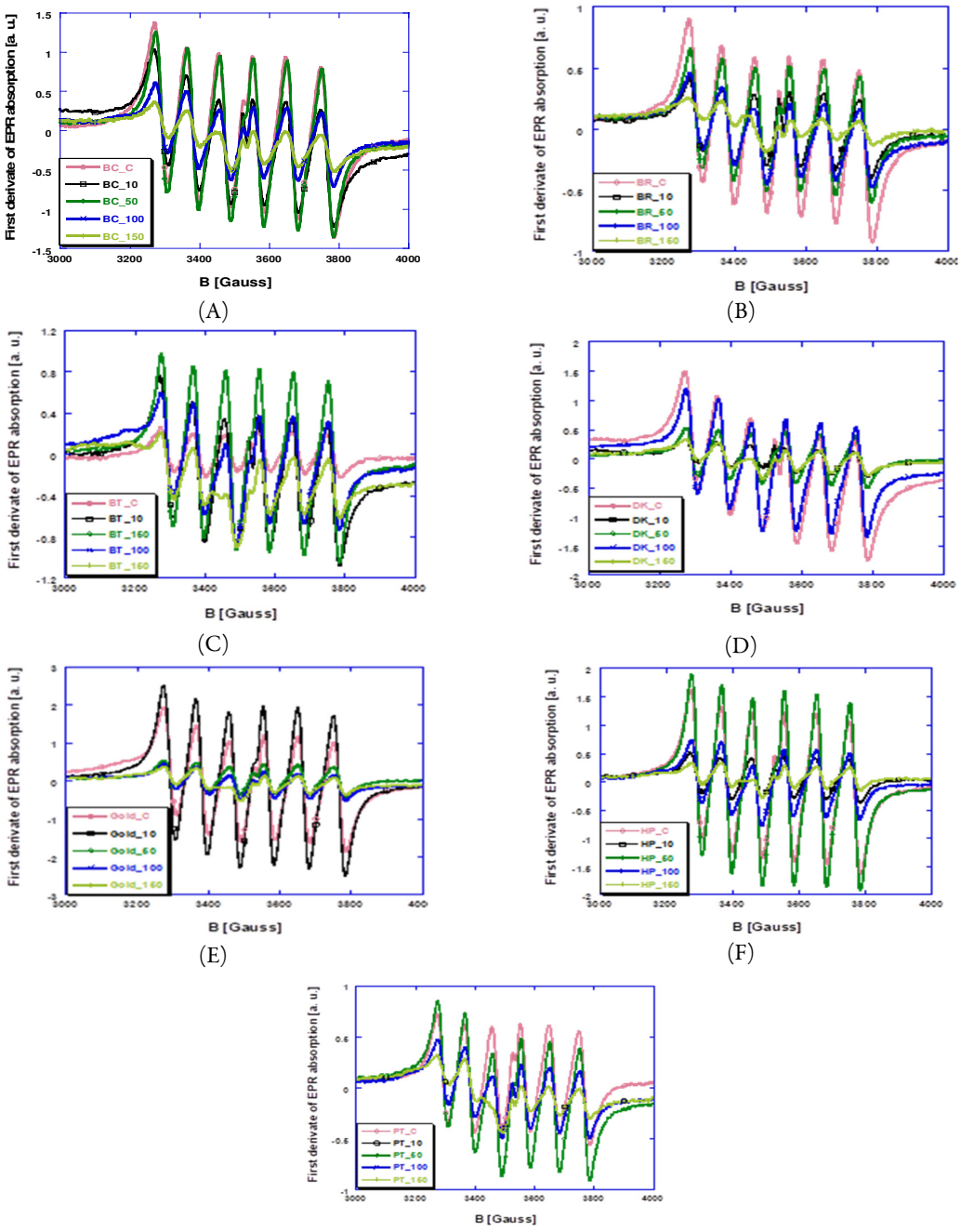


Figure 4. EPR spectra for seven highbush blueberry cultivars *in vitro*-grown on salt stress conditions: (A) 'Bluecrop', (B) 'Blueray', (C) Briggita Blue, (D) 'Duke', (E) 'Goldtraube', (F) 'Hortblue Petite', and (G) 'Patriot'. For inducing the salinity stress in culture media [Woody plant medium (WPM) + 100 mg/l Sequestren 138 + 1 mg/L zeatin (Z) + 4 g/L Plant agar, pH = 5] were added 0, 10, 50, 100, and 150 mM NaCl

Discussion

One of the major abiotic factors influencing irrigated regions and agricultural crop yield is salinity (Yildirim *et al*., 2015) Plant growth may be inhibited by salts in the soil water for two reasons. First off, plants that have salt in their soil solution have a harder time absorbing water, which slows down their pace of growth. This is known as the salinity's osmotic or water-deficit impact. Second, the cells in the transpiring leaves will be harmed if too much salt enters the plant through the transpiration stream, which could result in further growth decreases (Parihar *et al*., 2015).

The accumulation of salts in soil or growing media affects various plant growth and development processes. Like many perennial fruit crops, blueberries have low salt tolerance, and understanding the threshold at which salts impede crop growth is essential. According to Bryla *et al*. (2021), it is currently uncertain whether blueberry varieties differ in their saline tolerance. As a result, the hypothesis that the salinity limitations for the two cultivars of highbush blueberries studied would differ depending on the type of salt was proposed.

To characterize how different blueberry cultivars respond to salinity stress, seven blueberry cultivars were exposed to 10, 50, 100, and 150 mM NaCl for 10 weeks in *in vitro* conditions. The *in vitro* cultures treated with NaCl showed statistical differences in the number of shoots, shoot length, fresh and dry weight, and water content even at the lowest salt concentration in the culture medium, 10 mM NaCl, while the concentration of 150 mM greatly affected the measured growth parameters. This highlights that the highbush blueberry is much more sensitive to salinity stress compared to other woody species subjected to *in vitro* salinity stress. For example, olive shoots showed growth reductions under 200 mM NaCl (Bashir *et al*., 2021), and sea buckthorn shoots showed reductions under 250 mM NaCl (Urbinati *et al*., 2020).

Our study revealed a notable and consistent reduction in the number of shoots per explant as the NaCl concentration increased. The observed trend exhibited a linear decline, indicating a dose-dependent response of blueberry plantlets to elevated salinity levels. This phenomenon suggests a heightened sensitivity of the *in vitro*-cultivated blueberry plantlets to increasing NaCl concentrations, emphasizing the necessity for precise salinity control in their growth environment.

However, a difference was observed among the seven cultivars regarding the number of proliferated shoots under different concentrations of NaCl. Considering all salt concentrations, it was observed that 'Brigitta Blue' was the most affected, having the highest number of shoots per explant under non-saline stress conditions (13.56), with the number of shoots decreasing by 2 times under 50 mM NaCl (10.66), 5 times under 100 mM NaCl (60.20), and 117 times under 150 mM NaCl (0.12). These results are in accordance with those reported by (Bashir *et al*., 2021), who showed that the number of proliferated shoots decreased with increasing NaCl concentration for four olive genotypes cultivated *in vitro* under three NaCl concentrations. Similar results were also reported for *Cicer arietinum* grown *in vitro* under different NaCl concentrations (Aasim *et al.*, 2023). A considerable decrease in the number of shoots was recorded with increasing salt concentration in the *in vitro* culture of spinach from 6.0±0.0 (control) to 1.0±0.0 (300 mM NaCl) (Muchate *et al*., 2019).

The investigation into the influence of sodium chloride (NaCl) concentrations on shoot length in the present study revealed a discernible impact, with a concentration-dependent effect. Similarly, to the number of proliferated shoots per explant, the length of shoots was the most affected by all salt concentrations in the 'Brigitta Blue' (Figure 1 K-O, Figure 2 B). In culture media supplemented with 50, 100, and 150 mM NaCl, the shoot length of this cultivar decreased by 3.21 times, 4.92 times, and 70 times, respectively, from 3 cm (0 NaCl) to 5 mm (150 mM NaCl). The negative impact of NaCl concentrations on shoot length is a common characteristic of plants and has been confirmed by other studies (Dogan, 2020; Bimurzayev *et al*., 2021; Kumar *et al*., 2021). For instance, salt concentrations in the culture medium have caused the inhibition of shoot height growth in three genotypes of Solanum (*S. melongena*, its wild relative *S. insanum*, and their interspecific hybrid), when grown in the presence of 200 and 400 mM of NaCl (Ortega-Albero *et al*., 2023). Regarding shoot length, our study identified the 'Bluecrop' and 'Patriot' cultivars as exhibiting better resistance to stress

induced by the lowest concentration of NaCl. Thus, under 10 mM NaCl, the difference in shoot length was not statistically significant compared to the salt-free control for these cultivars (Figure 2B). Under high salt concentrations, the 'Goldtraube' cultivar exhibited the highest resistance, particularly under 100 mM NaCl, where the shoot length was halved compared to the control. In comparison, the shoot length decreased by 13 times in 'Blueray', 7 times in 'Patriot', and 6 times in 'Duke'. This insight might prove valuable for selecting cultivars suitable for regions with saline soil conditions, offering practical implications for optimizing agricultural practices under such environmental constraints.

In our study, blueberry shoots *in vitro* exposed to different concentrations of NaCl exhibited a significant decrease in FW, particularly evident at high salt concentrations (100 and 150 mM NaCl). Similar to the number and length of shoots, 'Brigitta Blue' was the most affected, with FW decreasing by 106 times in the medium with 150 mM NaCl compared to the control. Consistent with these findings, various studies have reported a decrease in FW in different species subjected to salinity stress *in vitro* (Hannachi *et al*., 2021; El-Mahdy *et al*., 2022; Radi *et al*., 2023). Additionally, the negative effect of salinity on FW has been demonstrated through numerous in vivo studies on tomatoes with 75 mM NaCl concentration (Win *et al*., 2018) on okra seed-lings at mM 100 NaCl concentration (Wang *et al*., 2019) at apple rootstock (Matsumoto and Kobayashi, 2020) and sour cherry at 60 mM NaCl (Papadakis *et al*., 2018).

Saline stress is usually accompanied by changes in water content (Hao *et al*., 2021). Previous studies, carried out both in vivo and *in vitro*, showed that in most species WC decreased with increasing salt concentrations (İzgü *et al*, 2023; Parihar *et al*., 2015; Chourasia *et al*., 2022; Balasubramaniam *et al*., 2023). In contrast, Gisela 5 shoots cultured *in vitro* exhibited a different behavior, as treatments with 50, 100, and 150 mM NaCl had no effect on WC (Erturk *et al*., 2007). Contrary to these results, blueberry shoots exposed to 50 and 100 mM NaCl had higher WC compared to the control. Under 150 mM NaCl, WC values were lower than under 0 mM NaCl, consistent with results reported in other species. The presence of higher water content in blueberry shoots grown under 50 and 100 mM NaCl could be attributed to the phenomenon of hyperhydricity observed in some shoots under high salt concentrations. Hyperhydric shoots have elevated moisture due to the accumulation of water in intercellular spaces (Polivanova and Bedarev, 2022).

Salt tolerance, as expressed by STI (Table 2), had a general tendency to decrease with increasing salinity levels. Similar results were observed at *Solanum tuberosum*, *Oryza Sativa*, *Zea mays* and *Fragaria* x *ananassa* cvs. (Carpıcı *et al*., 2009; Chunthaburee *et al*., 2016; Denaxa *et al*., 2022; Zaki and Radwan, 2022a).

In our study, ITS values calculated based on the number of shoots, shoot length, FW (fresh weight), and DW (dry weight) confirm that 'Goldtraube' was the most tolerant among the cultivars under 50, 100, and 150 mM NaCl conditions. Under low salinity (10 mM NaCl), 'Bluecrop' was the most salt-tolerant, presenting the highest ITS values. Additionally, ITS confirmed that 'Brigitta Blue' was the most sensitive to salinity stress, exhibiting the lowest values.

The evaluation of blueberry's response to salinity stress using the McKinney Index (MKI), which indicates the level of chlorosis and necrosis of shoots, shows a significant increase in necrosis with increasing NaCl concentration across all cultivars. MKI values of zero indicate that all varieties had green leaves and shoots at 0 and 10 mM NaCl, while under the treatment of 150 mM NaCl, necrosis affected a large number of shoots, which is why MKI was not calculated. Additionally, MKI confirms that the most resistant cultivar was 'Goldtraube', while the most affected by salinity stress was 'Brigitta Blue', with MKI values of 3.2 and 5.8 under 100 mM NaCl, respectively.

According to the literature, EPR Spectroscopy can be used to observe the main paramagnetic components (Fe3+, free radicals, Mn2+) in plants (Csillag and Damian, 2016). The superimposed sextet is characteristic of the Mn (II) ion which is partially unlimited by proteins (Reed and Cohn, 1970; Reed *et al*., 2002; Villafranca *et. al*., 2002). Moreover, the analysis of the EPR signal showed distortions at the level of small magnetic fields. These distortions appear when asymmetries of the magnetic field of the Mn (II) binder are present.

The specific sextet of Mn (II) especially in biological samples through the 6 hyperfine lines centered is around g = 2 (Beinert and Palmer, 1965). In this study, EPR spectroscopy was used to analyze how the hyperfine structure of Mn (II) was influenced by the effect of salinity in fresh blueberry leaves. Mn (II) is involved in the catalysis of water scission, the evolution of oxygen in the photosystem (II) - which is a vital part of our planet's survival. It is also a micronutrient involved in the photosynthesis of green plants and cyanobacteria (Debus, 1992; Brudvig *et al*., 2009).

In our study, the EPR spectra characteristic of the presence of the Mn (II) complex at different concentrations of the stress factor NaCl show an important limitation of the conformational ways of binding manganese ions with the increase of the NaCl concentration in the studied biological samples. Therefore, with the increase of the stress factor concentration, we notice an important change in the sextet characteristic of the Mn (II) complex, by solving the resulting EPR spectrum.

As shown in Figure 4A, the broad and strong signal (EPR spectrum of 'Bluecrop') is a specific EPR signal of a paramagnetic species of transition metals in a powder sample (Morsy and Khaled, 2002). It is already known that Mn (II) binds to the protein configurations through a very large number of different configurations and paramagnetic states, therefore a signal of a very noisy sextet is specific to each biological system normally found in nature. The presence of a stress factor (NaCl in this case) strongly influences the multitude of configurations and paramagnetic states specific to the Mn (II) ion (Csillag and Damian, 2016). This may explain why the manganese ion signal becomes resolved as the stress factor increases. Samples 'Bluecrop' and 'Duke' show the more resolved EPR spectra of the super fine sextet of Mn (II) at different concentrations of the stressor NaCl which can be attributed to the free flipping of Mn (II). On the other hand, 'Goldtraube' presents the most unresolved spectra of the Mn (II) hyperfine structure under all salt concentrations (Figure 4E). This aspect indicates that the multitude of possible binding conformations of Mn (II) is little influenced by the presence of salt, therefore 'Goldtraube' is the most tolerant to saline stress. The EPR results are in agreement with ITS and MKI which also showed the high salinity tolerance of 'Goldtraube'.

Conclusions

The results of this study showed that salinity stress induced in blueberry cultures *in vitro* by adding NaCl to the culture medium reduced the number of shoots obtained and shoot length in all studied blueberry cultivars and under all salt concentrations. The EPR spectra of fresh leaves showed a significant change in the characteristic Mn(II) sextet. Both the morphological changes and stress tolerance indices and the EPR spectra showed that 'Goldtraube' showed the highest tolerance to saline stress.

Authors' Contributions

Conceptualization, DC and SM; methodology, DC, VP, and SM; software, DC, FAA, and SM; investigation, DC, VP, MH, FAA, and S.; resources, DC; data curation, DC, VP, and SM; writing-original draft preparation, SM; writing-review and editing, SM, DC, VP, MH and CIB. All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

Acknowledgements

This work was supported by the Ministry of Research, Development, and Innovation, developed with the support of UEFISCDI (Project No. 14 PFE-2022-2024) and Internal grant of UASVMCN, number 18347/2019.

Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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