SUMMARY OF THE PhD THESIS

Resilience of some highbush blueberry (*Vaccinium corymbosum* L.) cultivars to abiotic stresses under in vitro conditions

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Introduction

Highbush blueberry (*Vaccinium corymbosum* L.) is the most commonly cultivated, commercially important, and biologically valuable species of the genus *Vaccinium*. In recent years, blueberries have become increasingly popular among consumers worldwide due to their flavor, high nutritional value, and health benefits (RUZIĆ et al. 2012, MAZUREK et al., 2021; MUÑOZ-FARIÑA et al., 2023; SHI et al., 2023).

At the same time, highbush blueberry is one of the most sensitive species to water shortages in soils due to its superficial root system, that lacks root hair that penetrate the soil only up to 60 cm in soil (BRYLA & STRIK, 2007; SANDOVAL et al., 2024). Being in need of irrigation even in humid climates Also soil salinity is becoming an increasing problem for the production of highbush blueberry (BRYLA et al., 2021; MURALITHARAN et al., 1992), The blueberry being particularly vulnerable to the high level of salt in the soil. Therefore, the breeding of varieties more tolerant to drought and salinity can contribute to the efficiency of commercial plantations in increasingly severe climatic conditions.

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). Due to these drawbacks, more reliable selection techniques have been developed using in vitro culture technology (in vitro screening). To induce water stress, culture media are supplemented with various high molecular weight osmotic substances such as polyethylene glycol (PEG) and salt stress is simulated by adding NaCl. The use of in vitro cultures offers a number of advantages compared to the traditional methods of assessing tolerance to drought and salinity in the field, such as: obtaining results quickly, eliminating climatic disturbances, uniformity of experiments, performing a large number of experiments simultaneously and in a small space as well as economy of manpower, soil or water.

The in vitro screening condition is considered beneficial compared to field screening, although the stress effect may not be replicated at all stages of development through this method. Preliminary reports can predict the plant response to stress, and subsequent validation of responses to stress can be conducted under field conditions (SAHU et al., 2023). Therefore, this method is used, in particular, to make an early assessment of the response of genotypes to different types of stress and the collected data can be the basis of future breeding programs.

In order to understand the response of plants to abiotic stress conditions, a

series of interdisciplinary studies (physiological, biochemical, genetic, molecular, physical, etc.) are addressed.

Along with the morphological changes of plants under abiotic stress, some biochemical parameters such as photosynthetic pigments, antioxidant compounds, oxidative stress markers or osmolytes can define the response of plants to drought stress (COŞKUN, 2023; EISA et al., 2023; HABUŠ JERČIĆ ; et al., 2023) The applications of electron paramagnetic resonance (EPR) spectroscopy in plant research enable 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). EPR spectroscopy allows the investigation of biological tissues due to the presence of various paramagnetic centers such as manganese (Mn), iron (Fe), and copper (Cu), which are found in enzymes and other biological structures.

Through this study, the adaptation capacity of highbush blueberry to hydric and saline stress was tracked using in vitro cultures and the evaluation of the obtained morphological and biochemical responses provides important data to understand the mechanisms by which these varieties can tolerate these abiotic factors.

Structure of the doctoral thesis

The doctoral thesis includes a total of 136 pages and is structured in two parts:

- State of the art – 24 pages
- Original research – 61 pages

The first part (state of the art) has 1 chapter which present the current state and perspectives of the blueberry industry alongside with blueberry growing conditions.

The second part of the thesis (original research) includes 6 chapters that presents the studies, objectives, conclusions and recommendations, as well as the originality and innovative contributions of the research.

The PhD thesis includes a total of 8 tables, 19 figures and 219 bibliographic references.

Results and discussion

The research carried out as part of the doctoral thesis was published in 3 articles published in ISI journals and is presented in the published form in chapters 3,

4 and 5.

Chapter 3 The objective of the present chapter was to evaluate the effects of drought on plant growth and development in five highbush blueberry cultivars (Bluecrop, Brigitta Blue, Duke, Goldtraube and Hortblue Petite, under drought stress conditions induced by different PEG 6000 concentrations added to the in vitro culture medium.

The obtained results showed that increasing PEG concentration resulted in a decrease in photosynthetic pigments for all the varieties studied. It is noteworthy that, in terms of these parameters, Goldtraube was the most sensitive to water stress induced by PEG.

Shoots from PEG free culture media (for all varieties) showed the highest level in all photosynthetic pigments. Showing that the composition of the photosynthetic pigments is affected by lack of water therefore there is strong connection between the general health of the plant and the levels of chlorophyll a and b and carotenoids. The highest values were recorded in Bluecrop: 1.91 ± 0.04 mg/g FW Chlorophyl a, 0.75 ± 0.04 mg/g FW Chlorophyl b and 1.19 ± 0.02 mg/g FW for carotenoids.

The aim of the study found in **chapter 4** 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. 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.

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

Both the morphological changes and stress tolerance indices and the EPR spectra showed that 'Goldtraube' showed the highest tolerance to saline stress.

In **Chapter 5** the objective of the study was to evaluate the morphological and biochemical responses in in vitro blueberry cultivar Hortblue Petite shoots under drought stress conditions induced by PEG 6000. The most significant morphological and biochemical parameters that responded to drought stress in our study were: shoot length, shoot number, content of photosynthetic pigments, proline, malondialdehyde,

and hydrogen peroxide. A reduction in proliferated shoot length and an increase in shoot number were observed on the WPM + 1 mg/l Z culture medium supplemented with 10, 20, 30, 40, and 50 g/l PEG. The content of photosynthetic pigments decreased, while the content of proline, malondialdehyde, and hydrogen peroxide increased with the increasing concentration of PEG 6000 in the culture medium.

The results obtained suggest that the in vitro screening technique of highbush blueberries for drought tolerance, utilizing PEG as a stress agent, could serve as an alternative method for the early selection of drought-resistant cultivars. However, for validation, field research is recommended in line with practices for other species.

General conclusions and recommendations

The present thesis is based on four research studies resumed as follows:

Chapter 1 presents the current state and perspectives of the blueberry industry the challenges of blueberry growing due to biotic and abiotic factors and recommended growing conditions.

Chapter 3 presents a study that tested the potential drought tolerance of five blueberry cultivars (Duke, Bluecrop, Brigitta Blue, Goldtraube, and Hortblue Petite) subjected to drought-like stress conditions created by adding five concentrations of PEG 6000 (10, 20, 30, 40, and 50 g/l PEG.) to the in vitro culture media, examining their morphological, physiological, and biochemical parameters. The obtained results showed that increasing PEG concentration resulted in a decrease in photosynthetic pigments for all the varieties studied.

Chapter 4 presents an investigation of the salinity tolerance of seven blueberry cultivars (Bluecrop, Blueray, Brigitta Blue Duke, Goldtraube, Hortblue Petite, and Patriot) in in vitro conditions. Salt stress conditions were created by adding four concentrations of NaCl (10, 50, 100, and 150 mM), to the in vitro culture media. The monitored parameters were the number of shoots obtained/explant, shoot length, fresh weight, dry weight, and water content. For an in-depth analysis, the indexes were calculated: stress tolerance index (ITS), and McKinney index (MKI). Fresh leaves obtained in vitro were used for the electron paramagnetic resonance (EPR) measurements to assess the behaviour of the seven blueberry cultivars under salt stress using electron paramagnetic resonance (EPR) spectroscopy.

Chapter 5 presents how biochemical markers involved in plant resistance to abiotic stress can provide data on the drought stress response mechanisms of the Hortblue Petit blueberry cultivar. The most significant biochemical parameters that responded to drought stress were: the content of photosynthetic pigments (Chl a, Chl b, Caro), total phenolic compounds (TPC), total flavonoid content (TFC), proline (Pro), malondialdehyde (MDA) and hydrogen peroxide (H_2O_2).

The main conclusions obtained from the research conducted were:

- In all studied blueberry cultivars, the following growth indicators—shoot length (SL), average number of shoots per culture pot (NS), proliferation rate (PR), and fresh weight (FW)—were influenced by drought stress simulated in vitro by PEG 6000.
- A single growth indicator (SL) decreased with increased PEG concentrations in all five varieties, indicating the damaging effects of PEG-induced drought stress.
- The increased PEG concentration resulted in a decrease in photosynthetic pigments for all the varieties studied.
- Goldtraube was the most sensitive to in vitro water stress induced by PEG 6000.
- The in vitro cultures of blueberry exposed to NaCl (10, 50, 100, and 150 mM) for 10 weeks 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).
- In all studied cultivars, the number of shoots per explant increased as the concentration of NaCl in the culture medium increased.
- 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, with the number of shoots decreasing under all NaCl concentrations.
- The investigation into the influence of sodium chloride (NaCl) concentrations on shoot length 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.
- 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). The most affected cultivar was Brigitta Blue.
- Salt tolerance, as expressed by the salt tolerance index (STI), had a general tendency to decrease with increasing salinity levels. The STI confirmed that Brigitta Blue was the most sensitive to salinity stress, showing the lowest values.
- 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.
- EPR spectra of fresh leaves from blueberry shoots grown in vitro under salt stress 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.

- The oxidative stress markers, malondialdehyde (MDA) and hydrogen peroxide (H_2O_2), showed a significant increase in blueberry shoots cultured on media exposed to all concentrations of PEG 6000.
- A positive correlation can be observed between proline accumulation and increasing drought levels
- In general, total flavonoids (TFC) and total phenolic content (TPC), content in the blueberry shoots were reduced at mild stress and increased at intense stress conditions. The highest level in both traits was obtained in the shoots treated with 50 g/l PEG.
- The studies carried out followed the capacity to adapt of the highbush blueberry to water and saline stress using in vitro cultures and the evaluation of the obtained morphological and biochemical responses provides important data to understand the mechanisms by which these varieties can tolerate these abiotic factors.
- The *in vitro* screening technique for highbush blueberries for drought tolerance and salt tolerance could serve as an alternative method for the early selection of drought-resistant varieties.

Recommendations

The obtained results show an increased sensitivity of blueberry plants grown in vitro to increasing concentrations of NaCl (salt stress) and PEG 6000 (drought stress), emphasizing the need for precise control of salinity and water deficit in their growth environment.

Using PEG 6000 and NaCl as an in vitro stressor is a convenient way to assess, under controlled conditions, the effects of abiotic stress on the growth and development of blueberry plants and could prove of great importance in reducing the time and cost of further studies.

It is recommended to conduct field research, following practices used for other species, to validate blueberry varieties proven to be tolerant to water and salt stress through in vitro screening.

Originality and personal contribution

For the first time, the ability to adapt to conditions of hydric stress induced in vitro with PEG 6000 of blueberry varieties Duke, Bluecrop, Brigitta Blue, Goldtraube, and Hortblue Petite was evaluated.

This is the first study in which in vitro culture technology is used to evaluate the salinity tolerance of Bluecrop, Blueray, Brigitta Blue Duke, Goldtraube, Hortblue Petite and Patriot blueberry varieties.

Electron paramagnetic resonance (EPR) spectroscopy was used for the first time to characterize the differences between blueberry cultivars exposed to different saline water stress conditions.

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