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Determination of the effects of drought stress on *Aronia melanocarpa* cv. Nero *in vitro* conditions

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Abstract
Drought stress is a significant threat to plant cultivation in arid and semi-arid regions, negatively affecting growth and leading to plant mortality. This study evaluated the *in vitro* drought tolerance of *Aronia melanocarpa* 'Nero' by exposing micropropagated plantlets to nutrient media containing different concentrations of PEG-8000 (0%, 1%, and 2%) during the rooting stage. Results showed that increasing PEG concentrations led to a reduction in survival, rooting, plantlet growth, and physiological parameters. The 0% PEG treatment resulted in the highest survival rate (95.83%), root number, and chlorophyll content, while the 2% PEG treatment significantly hindered these parameters. These findings indicate that *Aronia melanocarpa* 'Nero' is sensitive to drought stress, with reduced growth and physiological activity under higher PEG concentrations.
Keywords: Chlorophyll, Drought stress, *In vitro*, Micropropagation, Growth

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INTRODUCTION

Aronia is a berry like fruit found within the *Aronia* genus of the *Rosaceae* family. This fruit is native to North America and spread to Europe at the beginning of the twentieth century. There are many varieties of aronia. However, in our country, the 'Viking' and 'Nero' varieties are at the forefront. Fresh aronia fruits are rarely consumed due to their bitter taste. Its fruits are mostly used in the production of fruit juice, wine, tea, medicine and nutritional supplements. In addition, the anthocyanins in its content can be used as natural food coloring. It is an important fruit for human health with its high antioxidant content. Its use against cancer has become intense due to its high nutritional values and the natural content of polyphenolic substances that fight against free radicals (Kapci et al., 2013; Vagiri and Jensen, 2017; Sidor and Gramza-Michałowska, 2019; Yilmaz et al., 2021; Zhang et al., 2021).

Aronia is a hardy species that can be grown in areas where winter temperatures drop to -30-35°C, as well as being tolerant to frost. Production and fruit quality are higher in areas that can receive sunlight. It is not selective in terms of climate characteristics. However, they are sensitive to drought conditions. When drought conditions continue, they cannot withstand long periods and the quality of the fruits decreases (Negreanu-Pirjol et al., 2023).

Drought stress is one of the abiotic stress factors and negatively affects production, product yield and quality. Exposure to drought stress during the plant's growth process restricts growth and development. Drought stress can affect the morphological parameters of the plant as well as inhibit the functions of physiological parameters such as stomatal movements, photosynthesis and respiration. Plants primarily respond to changes in their external and internal structures under drought stress conditions. As the severity of drought stress increases, it can result in slow plant growth and plant death in the future (Mahajan and Tuteja, 2005; Yang et al., 2021).

Tissue culture methods are preferred *in vitro* due to the sustainability of the studies under controlled conditions and regardless of the vegetation period (Mese and Tangolar, 2019). Some chemical substances are used to determine tolerance to drought stress *in vitro*. Drought stress occurs by adding these stress agents to culture media at certain rates. Agents that cause drought stress *in vitro* are sorbitol, mannitol and polyethylene glycol (Simsek et al., 2018; Sattar et al., 2021; Bilir Ekbic et al., 2022).

The agent commonly used in tissue culture studies is polyethylene glycol. Polyethylene glycol (PEG) is a substance that can imitate drought stress in the early stages of plant growth. PEG has forms with a wide molecular weight between 200-10.000.000 g/mol. PEG is a substance with an osmotic structure. It reduces the water potential in the environment in which it is used. It is not a toxic substance. It is used as an artificial abiotic stress stimulant and is included in many stress studies (Toosi et al., 2014; Gullapalli and Mazzitelli, 2015; Herzberger et al., 2016; Othmani et al., 2021; Violita and Azhari, 2021; Pham Le Khanh et al., 2022).

There are no sufficient studies in the current literature on the resistance of 'Nero' variety to PEG-induced drought stress, so this research aims to fill the knowledge gap on this subject. Therefore, this study was conducted to investigate the effects of PEG-8000-induced drought stress on the morphological and physiological parameters of the plant *in vitro*.

MATERIALS AND METHODS

The study was conducted in the plant tissue culture laboratory of the Horticulture Department of the Faculty of Agriculture, Harran University. Shoots taken from 1-year-old plants grown in greenhouse conditions during the active development period were used. Micro cuttings with a single node belonging to the 'Nero' variety of *Aronia melanocarpa* [Michx.] Elliot were used as plant material. The micro cuttings were first subjected to a pre-sterilization process in the laboratory. In the pre-sterilization process, the micro cuttings were kept in detergent water for 10 minutes and then rinsed under tap water until completely cleaned. After this, the micro cuttings were taken into a sterile cabinet and first kept in 70% ethyl alcohol for 2 minutes, then in 10% NaCIO (sodium hypochlorite) solution for 10 minutes and then rinsed 3 times with sterile pure (Nas et al., 2023). After this, micro cuttings were transferred to tubes containing 20 ml of growth medium containing 3% sucrose, 1 mg L^{-1} GA_3, 0.8 g L^{-1} agar and MS.The pH of the nutrient medium was adjusted to 5.8 with 1N NaOH and 1N HCI. The plantlets obtained in the shoot propagation medium were transferred to the tillering medium containing 3% sucrose, 3 mg L^{-1} BAP, 1 mg L^{-1} kinetin, 1 ml L^{-1} PPM and 7 g L^{-1} agar (Almokar and Pirlak, 2018). When the sufficient number of plantlets was reached, the trial was designed. The trial was established at the rooting stage of the plantlets. In the study, three different doses of PEG-8000 were added to the rooting medium (Table 1). 3% sucrose, 7 g L^{-1} agar, 2 mg L^{-1} IBA and 0.5 mg L^{-1} NAA were added to the rooting medium (Polat and Eskimez, 2022). %0, %1 and 2% PEG-8000 was added to the nutrient medium. Only rooting hormone was added to the control group. 1 ml L^{-1} PPM (Plant preservative mixture) was added to prevent bacterial contamination that may occur in the nutrient medium (Babu et al., 2022; Kara et al., 2022; Ekinci et al., 2024). The plantlets transferred to the applications were kept in a growth chamber using white fluorescent lamps, at 16:8 hours photoperiod, 65-70% air relative humidity and 25±1°C temperature for 60 days (Saskin et al., 2022). The study was designed according to a randomized trial design with 3 replications and 10 plants in each replication.

Table 1. PEG-8000 concentrations in culture media.

Treatments	
T1	%0 PEG-8000
T2	%1 PEG-8000
T3	%2 PEG-8000

Figure 1. Development of plantlets exposed to PEG-8000-induced drought stress *in vitro*. T1: 0% PEG-8000, T2: 1% PEG-8000, T3: 2% PEG-8000

Measurements and Statistical Analysis

The study was terminated after 60 days. At the end of the application, morphological and physiological parameters such as survival rate, rooting rate, number of root, root length, shoot length, number of leaves, plant

fresh weight, plant dry weight, chlorophyll a, chlorophyll b, total chlorophyll were examined. The data obtained at the end of the applications in the study were compared with the LSD test at the $p \leq 0.05$ significance level using one-way variance analysis in the JMP Pro 13 statistical program (Gomez and Gomez., 1984). Hierarchical clustering analysis (HCA) was conducted via the Software R (Version 4.1.1, R Foundation for Statistical Computing, Vienna, Austria).

RESULTS AND DISCUSSION

Drought occurs due to global climate change and disruption of ecological balance. Drought seriously restricts agricultural production and reduces product yield and quality. At the same time, it can cause damage to the morphological development and physiological metabolism of the plant by preventing photosynthesis, respiration and stomatal movements of the plant during the plant growth process (Yang et al., 2021).

When the results obtained from the study were examined, no statistically significant difference was found in terms of survival rate under drought stress conditions (Table 2). The highest survival rate occurred in T1 (95.83%) and T2 (95.83%), and the lowest survival rate occurred in T3 (91.67%) applications. *Aronia melanocarpa* [Michx.] Elliot is not a very drought resistant species (Celik et al., 2022). The results obtained from our study support this opinion.

When the results obtained from the study under drought stress conditions were examined, the rooting rate was found to be statistically significant (Table 2; Figure 1). The highest rooting rate occurred in T1 (95.83%) and the lowest rooting rate occurred in T2 (37.50%) application. There were decreases in the rooting rate of plantlets in PEG applied nutrient media compared to the control. There are studies that decreased rooting rates with PEG applications. In a study conducted *in vitro*, when the responses to PEG induced drought stress in three different eggplant varieties were examined, it was stated that PEG applications reduced the rooting rate compared to the control (Zayova et al., 2017). In another study conducted under *in vitro* conditions, mannitol-induced drought stress conditions in five different potato cultivars significantly reduced the rooting rate compared to the control with applied mannitol concentrations, supporting our study (Dobránszki et al., 2003).

Table 2. Effect of PEG-8000 induced drought stress on morphological parameters.

Treatments	Morphological parameters			
	Survival Rate (%)	Rooting Rate (%)	Number of Root (per/plantlet)	Root Lenght (cm)
T1	95.83±7.22	95.83±7.22 a	24.08±1.81 a	5.29±0.49 a
T2	95.83±7.22	37.50±0.00 b	5.83±1.04 b	1.38±0.38 b
T3	91.67±7.22	50.00±12.50 b	4.50±1.00 b	1.25±0.15 b

*There is a 5% difference between means expressed with different letters in the same column (LSD)

When examined in terms of root number, a statistically significant difference was found between the applications. It was determined that the highest root number occurred in T1 (24.08 pieces/plantlet), and the lowest root number occurred in T3 (4.50 pieces/plantlet). With the decrease in the number of roots, the plant cannot get the substances it needs due to the decrease in the absorption of nutrients and minerals from the environment (Kocaçalışkan, 2003). In this study, the highest root number occurred in the control group and the decrease in the number of roots was observed with the increase in the severity of drought stress caused by PEG-8000, supporting this opinion. In some studies conducted under *in vitro* conditions, it was reported that the severity of drought stress increased with the increase in PEG concentration and subsequently a decrease in root development occurred (Mengesha et al., 2016; Gecene, 2020).

A statistically significant difference was found between the applications in terms of root length. The longest rooted plantlets occurred in the T1 (5.29 cm) application, and the shortest rooted plantlets occurred in the T3 (1.25 cm) application (Table 2; Figure 1). In studies conducted under drought stress conditions, the decrease in root length with increasing PEG concentrations is similar to the results obtained in our study. In studies conducted by some researchers, it was determined that root length decreased with increasing PEG concentration (Albiski et al., 2012; Meşe and Tangolar, 2019; Martínez-Santos et al., 2021). The obtained results support our study.

When the shoot length parameter was examined, a statistical difference was found between the applications. The longest shoots occurred in the T1 (4.28 cm) application, and the shortest shoots occurred in the T3 (0.79 cm) application. The main effect on the plant under drought stress conditions is the reduction of plant size (Salehi-Lisar et al., 2016). Optimum water is needed for plant growth and development. Cell growth in the plant is affected more by water scarcity than by cell division. Therefore, plant growth is inhibited by water deficiency under drought stress conditions (Sivritepe et al., 2008; Seleiman et al., 2019; Seleiman et al., 2021). Studies also support the results obtained from our study that shoot length decreases with increasing PEG concentration under drought stress

conditions. In a study conducted under *in vitro* conditions, five different PEG induced drought stresses were applied to different chickpea varieties, and it was reported that high concentrations of PEG negatively affected shoot length in chickpea varieties (Salma et al., 2016). In another study, four different PEG concentrations were applied to some wine grape varieties *in vitro* to determine their tolerance to PEG induced drought stress. The decrease in shoot lengths of all varieties with increasing PEG concentrations supports our study (Altıncı and Cangi, 2019).

A statistically significant difference was found between the applications in terms of leaf number. It was determined that the highest number of leaves occurred in T1 (24.67 pieces/plantlet), and the lowest number of leaves occurred in T3 (6.11 pieces/plantlet). In drought stress conditions, situations such as wilting of leaves, decrease in the number of leaves and change in leaf areas occur due to water deficiency (Yang et al., 2021). Plants tend to minimize water loss through transpiration by reducing the number of leaves depending on the severity and duration of drought (Jones and Cortlett, 1992). Similar results were obtained in the studies conducted. In a study conducted *in vitro*, five different PEG concentrations were applied to determine the tolerance of four grapevine rootstocks to drought stress. It was reported that as the PEG concentration increased, the number of leaves decreased (Mohsen et al., 2020). In another study, where morphological changes were examined against drought stress using five different almond varieties, the decrease in the number of leaves in all varieties occurred in parallel with the increase in drought stress, which supports our study (Zokaee-Khosroshahi et al., 2014).

Table 3. Effect of PEG-8000 induced drought stress on morphological parameters.

Treatments	Morphological parameters			
	Shoot Lenght (cm)	Number of Leaves (per/plantlet)	Plant Fresh Weight (g)	Plant Dry Weight (g)
T1	4.28±0.41 a	24.67±2.91 a	1.32±0.23 a	0.11±0.03
T2	1.28±0.19 b	10.89±0.69 b	0.45±0.09 b	0.09±0.01
T3	0.79±0.20 b	6.11±0.19 c	0.59±0.12 b	0.11±0.02

*There is a 5% difference between means expressed with different letters in the same column (LSD)

When the applications were examined in terms of plant fresh weight, a statistically significant difference was found between the applications. It was determined that the highest plant fresh weight occurred in the T1 (1.32 g) application, and the lowest fresh weight occurred in the T2 (0.45 g) application. Drought stress caused a serious decrease in the fresh weight of the plantlets. In order to prevent drying under drought stress conditions, stomata reduce their opening degree, and subsequently photosynthesis is affected by internal water deficiency. The amount of photosynthesis decreases with the decrease in the amount of carbon dioxide at the chloroplast level. The decrease in photosynthesis in plants under drought stress conditions results in the plants not storing sufficient amounts of dry matter in their bodies (Shangguan et al. 2000; Long et al. 2006; Yan et al. 2006; Lu et al., 2015). Results supporting this opinion were obtained in the studies. In a study, when the responses of five types of sunflower to drought stress were examined, it was reported that all varieties experienced significant decreases in plant fresh weight (Manivannan et al., 2007). In a study conducted to determine the drought tolerance of 5 kiwifruit species under *in vitro* conditions, the decreases in the fresh weight of plantlets under PEG induced drought stress conditions are parallel to our study (Zhong et al., 2018). When the applications were examined, no statistically significant difference was found between the applications in terms of plant dry weight. The highest plant dry weight was determined in T1 and T3 (0.11 g), and the lowest plant dry weight was determined in T2 (0.09 g).

Table 4. Effect of PEG-8000 induced drought stress on physiological parameters.

Treatments	Physiological Parameters		
	Chlorophyll a (mg/l)	Chlorophyll b (mg/l)	Total Chlorophyll (mg/l)
T1	2.24±0.46 a	0.79±0.16 a	3.03±0.61 a
T2	1.26±0.44 b	0.42±0.15 b	1.68±0.59 b
T3	0.60±0.18 b	0.19±0.06 b	0.78±0.24 b

*There is a 5% difference between means expressed with different letters in the same column (LSD)

When the applications were examined in terms of chlorophyll a, a statistically significant difference was found between the applications. It was determined that the highest chlorophyll a content occurred in T1 (2.24 mg/l), and the lowest chlorophyll a content occurred in T3 (0.60 mg/l). As a result of the applications, a statistically significant difference was found between the applications in terms of chlorophyll b. The highest chlorophyll b content was determined in T1 (0.79 mg/l), and the lowest chlorophyll b content was determined in T3 (0.19 mg/l). A statistically significant difference was found between the applications in terms of total chlorophyll amount. It was determined

that the highest total chlorophyll content was obtained from T1 (3.03 mg/l), and the lowest total chlorophyll content was obtained from T3 (0.78 mg/l). As the PEG concentration increased, significant decreases occurred in chlorophyll a, chlorophyll b and total chlorophyll content. Chlorophyll is the basic component of the chloroplast in providing photosynthesis. Chlorophyll content is in positive interaction with photosynthesis rate. The possibility of high photosynthesis depends on high chlorophyll content (Anjum et al., 2011; Nurcahyani et al., 2019). Under drought stress, stomata close, resulting in a decrease in photosynthesis rate. Since insufficient photosynthesis is performed, decreases in chlorophyll content occur (Mahajan and Tuteja, 2005). Under drought stress conditions, chlorophyll content decreases and changes occur in chlorophyll a and b contents. Studies have also yielded results that support our study (Hancı and Cebeci, 2014; Kabay and Şensoy, 2016).

HIERARCHICAL CLUSTERING ANALYSIS (HCA)

HCA is a clustering method that provides the organization of groups and samples between groups. The HCA result is presented with a tree-shaped drawing showing the organization of samples and the relationships of samples (Lee and Yang, 2009; Granato et al., 2018). HCA was performed using 11 different parameters in line with the applications performed in the study (Figure 2). Applications were clustered as I and II. Parameters were grouped in 5 different ways as A, B, C, D and E. T2 and T3 applications were in cluster I, T1 application was in cluster II. T1 application was composed of 0% PEG concentration. In T1 application, root length, root number, shoot length, plant fresh weight, rooting rate, total chlorophyll, chlorophyll a, chlorophyll b and leaf number parameters were positively affected. T2 and T3 applications were composed of 1% and 2% PEG concentration. PEG concentrations in T2 and T3 applications negatively affected morphological and physiological parameters. Plant dry weight in group A; survival rate in group B; leaf number, chlorophyll a and b in group C; rooting rate and plant fresh weight in group D; shoot length, root number and root length parameters in group E. In the color scale given on the right, red represents the highest value, yellow and orange colors represent intermediate values, and blue represents the lowest values. Scale values vary between approximately -1.0 and 1.0. It was observed that the parameters in cluster II have high values (especially in T1 application), while parameters in cluster I have medium and low values in T2 and T3 applications. The highest value (red) is in T1 application (%0 PEG) in cluster II. In cluster I, plant dry weight in T2 application (%1 PEG) and survival rate in T3 application (%2 PEG) have the lowest value (blue) (Figure 2).

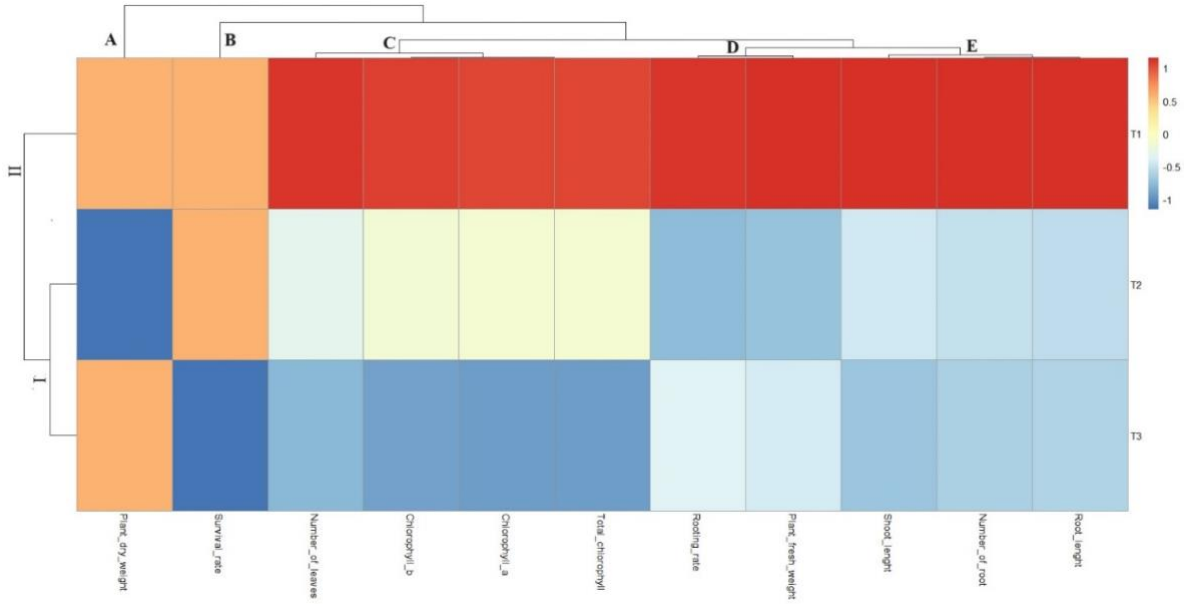

Figure 2. Heatmap for morphological and physiological parameters of plantlets exposed to PEG-8000 induced drought stress *in vitro*. T1: 0% PEG-8000, T2: 1% PEG-8000, T3: 2% PEG-8000. A, B, C, D and E represent the morphological and physiological parameters examined. I and II represent the applied PEG-8000 concentrations.

CONCLUSION

This study demonstrated that drought stress induced by PEG-8000 has significant effects on the growth and physiological parameters of *Aronia melanocarpa* 'Nero' under *in vitro* conditions. The findings indicate that increasing concentrations of PEG (1% and 2%) led to a decline in survival rate, rooting percentage, shoot and root length, leaf number, and chlorophyll content. These results highlight the sensitivity of *Aronia melanocarpa* 'Nero' to drought conditions, which limits its growth potential under water deficit.

In practical terms, these findings suggest that while *Aronia melanocarpa* is a valuable species due to its health benefits and antioxidant properties, its cultivation in areas prone to drought may require additional measures. The

use of irrigation systems or soil amendments to retain moisture could mitigate the negative effects of drought stress. Furthermore, selecting or breeding drought-tolerant cultivars of Aronia could be a promising strategy to enhance its resilience to water-limited environments.

Compliance with Ethical Standards

Peer-review

Externally peer-reviewed.

Declaration of Interests

The authors declared that there is no actual, potential or perceived conflict of interest in this research article.

Author contribution

The contribution of the authors to the present study is equal. All the authors verify that the text, figures, and tables are original. The authors read and approved the final manuscript.

Ethics committee approval

Ethics committee approval is not required.

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