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Research Article

The effect of sodium nitroprusside on the vegetative development of *Aronia melanocarpa* [Michx.] Elliot under *in vitro* conditions

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Abstract

Aronia is a berry fruit that has a significant amount of antioxidants. Both sexual and vegetative methods of propagation are available for aronia. Genetic diversity is present in the process of seed propagation. Therefore, clonal propagation techniques such as cuttings, basal shoots, and tissue culture are preferred. Plant tissue culture is a process that offers a high multiplication rate and the ability to produce clean plant material. This study aimed to stimulate the growth of plantlets by introducing several growth-promoting agents into the culture media. Specifically, the effects of a nitric oxide donor SNP (sodium nitroprusside) on the vegetative development of the plants were examined under *in vitro* conditions. Four distinct concentrations of SNP (0, 100, 200, and 300 μM) were administered to promote vegetative development. The survival rate (%), rooting rate (%), root number (per plantlet), root length (cm), leaf number (per plantlet), shoot length (cm), plant fresh weight (g), and plant dry weight (g) of the plantlets were estimated on the 60th day of growth in the nutrient medium under *in vitro* conditions. The survival rate was 100% for the applications of SNP at concentrations of 100 μM and 200 μM. The 0 μM treatment had the highest root number, the 100 μM treatment had the longest root, and the 200 μM treatment had the highest values for shoot length. The 0 μM treatment provided the highest values for plant fresh weight, while the 0 and 100 μM applications resulted in the highest plant dry weight values. The study suggested that an increased concentration of SNP causes a toxic effect.

Keywords: *Aronia melanocarpa* [Michx.] Elliot; *in vitro*; regeneration; sodium nitroprusside; vegetative development

Introduction

Aronia is berry fruit within the Rosaceae family, belonging to the *Aronia* genus. This species originated in North America and has spread to other parts of the world since the 1920's. Aronia's high antioxidant content makes it not only suitable for fresh consumption but also for use in the food and pharmaceutical industries

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(Engin *et al.*, 2018; Poyraz Engin and Boz, 2019; Özder, 2021). It contains a natural blend of antioxidant polyphenolic compounds that play a significant role against free radicals, making it recommended for the prevention of cancer and metabolic diseases in recent times (Yılmaz *et al.*, 2021). It is referred to as a superfruit due to its high vitamin values (Michalak, 2015; Şahin and Erdoğan, 2022).

Aronia is a self-pollinating species, requiring no pollinator in orchard cultivation. It can be easily propagated through both seeds and vegetative methods. However, micropropagation is preferred for the production of disease-free plant material, high multiplication rates, and the ability to produce without being dependent on the vegetation period, all under suitable laboratory conditions (Almokar and Pırlak, 2018; Şaşkın *et al.*, 2022).

Micropropagation is a tissue culture method where healthy plant shoot bud is transferred to a culture medium during the active or dormant period and the bud transforms into a plant tissue. In this method, healthy young shoots are taken from the plant to be transferred to the culture medium. Micro shoots are initially transferred to the shoot multiplication medium. The aim at this stage is to ensure the growth of the micro shoot and then proceed to the bud formation stage. In this culture medium, plant growth-regulating hormones called cytokinins are used during both the shoot acquisition and the bud formation stages. The use of cytokinins promotes cell division, shoot formation, and development. In the rooting stage, auxins are used. Auxins promote cell elongation, cell division, tissue swelling, and root formation. Obtaining a large number of plants in micropropagation and rooting of the obtained seedlings are crucial. The mentioned cytokinins and auxins are essential plant growth regulators used to obtain a large number of plants and for rooting. However, in recent times, various plant growth-promoting molecules have been used (George *et al.*, 2008; Sezgin and Dumanoğlu, 2009; Kara *et al.*, 2022; Şaşkın *et al.*, 2022).

In vitro micropropagation, shoot and root growth are important, and plant growth-regulating molecules support vegetative development. Sodium nitroprusside (SNP), a nitric oxide donor, is one of the widely used plant growth-regulating molecules in plant tissue culture protocols under *in vitro* conditions. SNP has a growth-promoting effect on plants. Using SNP under *in vitro* conditions results in a high rate of shoot development and root formation in a short period. When this molecule is used in combination with auxins and cytokinins, it positively influences plant growth and development. The use of SNP increases shoot and root formation, parallelly enhancing the success of micropropagation (Scherer and Holk, 2000; Tan *et al.*, 2013; Khurana *et al.*, 2014; Sundararajan *et al.*, 2022). The roots and leaves of the plant play a crucial role in absorbing essential nutrients. An increased number of roots allow the plant to access water, organic matter, and nutrients more easily and rapidly. A higher leaf count contributes to increased photosynthesis, positively affecting the plant's development through enhanced nutrient production (Figure 1). Considering this information, this study was designed due to the positive effects of the SNP molecule on the plant's vegetative development.

The study aimed to answer the question, 'Did the application of SNP increase root formation, shoot growth, and leaf development in the plantlets?' This study investigated the effects of four different doses of sodium nitroprusside (0, 100, 200 and 300 μM) application on the growth parameters (survival rate (%), rooting rate (%), number of roots per plantlet, root length (cm), number of leaves (pieces/plantlet), shoot length (cm), plant fresh weight (g), and plant dry weight (g) of *Aronia melanocarpa* [Michx.] Elliot under *in vitro* conditions.

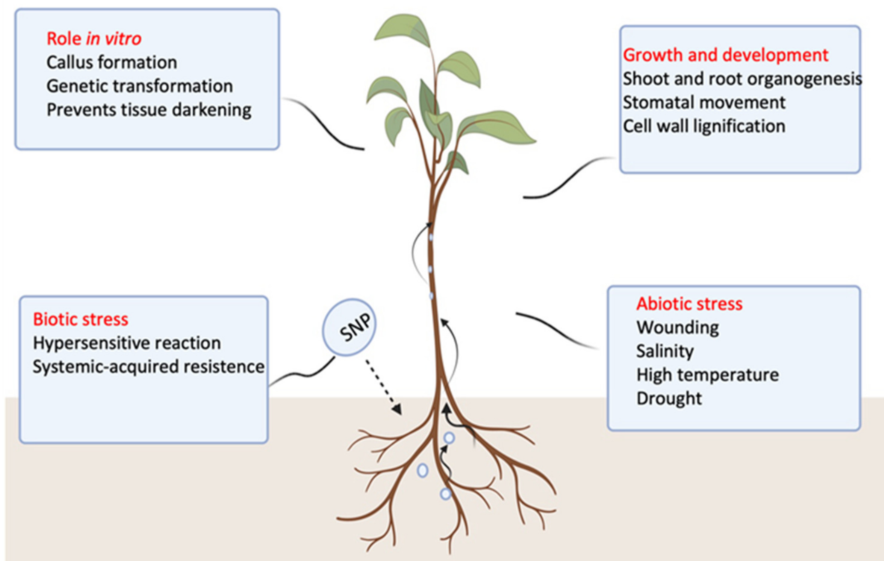

Figure 1. Role of SNP in various plant growth and development processes

Materials and Methods

The study was conducted at the Plant Tissue Culture Laboratory of Department of Horticulture, Faculty of Agriculture, Harran University, Sanliurfa, Türkiye. The 'Nero' variety of *Aronia melanocarpa* [Michx.] Elliot was chosen as the plant material. The micro cuttings were taken from the variety plot owned by the GAP Agricultural Research Institute. The micro cuttings were initially subjected to pre-sterilization. They were soaked in soapy water for 10 minutes and then rinsed with tap water. After pre-sterilization, the micro cuttings were placed in a sterile cabinet, immersed in 70% ethyl alcohol for 2 minutes, and then soaked in a 10% NaClO (sodium hypochlorite) solution for 10 minutes. After this process, the micro cuttings were rinsed three times with sterile distilled water. Following the sterilization process, the micro cuttings were transferred to the shoot medium. The shoot medium consisted of solid MS nutrient medium with 3% sucrose, 1 mg/l GA_3, 0.8 g/l agar (Nas *et al*., 2023). The pH of the nutrient medium was adjusted to 5.8. To prevent bacterial contamination in the nutrient medium, 1 ml/l of Plant Preservative Mixture (PPM) was added (Ak *et al*., 2021; Babu *et al*., 2022; Kara *et al*., 2022; Ekinci *et al*., 2023). The shoots were transferred to a tillering medium containing 3% sucrose, 3 mg/l BAP, 1 mg/l kinetin, 1 ml/l PPM, and 0.7 g/l agar for proliferation. The experimental design was arranged when the desired number of shoots was reached. Four different doses of SNP were added to the rooting medium in the study (Table 1). The culture medium included 3% sucrose, 0.6% agar, 1.5 mg/l IBA, and sequentially 0, 100, 200, and 300 µM SNP. The control treatment received only the rooting hormone. The micro cuttings transferred to the culture medium were kept in a growth chamber with white fluorescent lights for 16 hours of light and 8 hours of darkness, at 55-60% relative humidity, and a temperature of 25±1 °C for 60 days. The study was set up in a randomized complete block design with three replications, each consisting of 10 plants.

Table 1. Experiment plan

Treatments	
T1	1.5 mg/L IBA + 0 μM SNP
T2	1.5 mg/L IBA + 100 μM SNP
T3	1.5 mg/L IBA + 200 μM SNP
T4	1.5 mg/L IBA + 300 μM SNP

Figure 2. The effect of different doses of SNP application on the vegetative development of the plant under *in vitro* conditions. SNP treatments: T1=0 μM SNP; T2=100 μM SNP; T3=200 μM SNP; T4=300 μM SNP

Measurements and statistical analyses

The application was concluded after 60 days. The survival rate, rooting rate, root number, root length, leaf number, shoot length, plant fresh weight, and plant dry weight of the plantlets continuing their development under *in vitro* conditions were examined. At the end of 60 days, the root and shoot lengths of the plants removed from the nutrient medium were measured using a ruler. The numbers of roots and leaves were determined by counting. The survival rate and rooting rate was determined as percent. Plant fresh weight was determined using a precision balance with a sensitivity of 0.001 g. The plants with determined fresh weights were dried in an oven at a temperature of 70 °C for a duration of 72 hours. After drying, the plants were weighed again using a precision balance with a sensitivity of 0.001 g to determine the plant dry weight (Dikmetaş, 2020). The data were subjected to analysis of variance (ANOVA) using the Gomez and Gomez (1984) technique. Least Significant Difference (significance level 0.05) was used to determine significant differences between applications. JMP Pro 13 software was used for Least Significant Difference Test. Hierarchical clustering analysis (HCA) and principal component analysis (PCA) are commonly used statistical tools for grouping data and determining similarities between samples (Granato *et al*., 2018). Hierarchical clustering analysis (HCA) and principal component analysis (PCA) were conducted via the Software R (Version 4.1.1, R Foundation for Statistical Computing, Vienna, Austria).

Results and Discussion

Survival rate (%)

There was no statistically significant difference among the treatments in terms of the number of surviving plants. The highest survival rate was observed at 100% in T2 and T3, while the lowest survival rate was 95.56% in the T4 application (Table 2). Contaminations occurring in the culture media can directly damage the vegetative development of plants (Niedz and Bausher 2002; Babu *et al*., 2022). However, in this study, the absence of contamination positively affected the survival rate, while an increased SNP dose (T4) resulted in a 5% decrease. Our findings align with other studies indicating that plants exposed to stress under *in vitro* conditions experience a decrease in survival rate, and the application of SNP significantly increases the survival rate of plants (Ghadakchiasl *et al.*, 2017).

Rooting rate (%)

When examining the rooting rate, a statistically significant difference was found among the treatments. The highest rooting rate was observed at 100% in the T2 application, while the lowest rooting rate was 73.33% in the T4 application (Table 2).

In micropropagation performed under *in vitro* conditions, the ability of explants transferred to the culture medium to produce shoots and roots at a high rate and in a short time plays an important role. SNP applications under *in vitro* conditions promote rooting. Studies have elucidated the role of NO in main root development, including capillary root formation. Our findings in previous studies examining the rooting status of plants are supported by studies conducted on soybean seedlings under *in vitro* conditions (Pagnussat *et al.*, 2003; Correa-Aragunde *et al.*, 2006; Sundararajan *et al.*, 2022). Examining the rooting status of plants in previous studies, our findings are supported by research on soybean seedlings under *in vitro* conditions. In conditions of NaCl-induced salinity stress, SNP applications positively affected the rooting of soybean seedlings, and the combination with IBA further increased the rooting, as shown in our study (Karthik *et al.*, 2019). However, when used at high concentrations, it can exhibit a toxic effect, leading to adverse outcomes (Pradhan *et al.*, 2020). In our study, it was observed that T4 (300 µM SNP) application had a toxic effect.

Table 2. The effect of applied SNP under *in vitro* conditions on growth parameters

SNP treatments	Survival rate (%)	Rooting rate (%)	Number of roots (pieces/plantlet)	Root length (cm)
T1=0 µM SNP	97.78±3.85	80.00± 6.67 b	15.94± 1.69 a	8.23± 0.29 b
T2=100 µM SNP	100.00±0.00	100.00± 0.00 a	15.39± 1.18 a	9.24± 0.46 a
T3=200 µM SNP	100.00±0.00	97.78± 3.85 a	13.56± 0.69 a	9.27± 0.68 a
T4=300 µM SNP	95.56±7.70	73.33± 6.67 b	8.56± 0.51 b	3.03± 0.51 c
LSD p≤ 0.05	Ns	4.71	1,04	0.36
SNP treatments	**Number of leaves (per plantlet)**	**Shoot length (cm)**	**Plant fresh weight (g)**	**Plant dry weight (g)**
T1=0 µM SNP	13.42±2.93	1.81± 0.14 b	1.74± 0.13 a	0 12± 0.02 a
T2=100 µM SNP	14.83±2.40	2.35± 0.28 a	1.30± 0.11 b	0 11± 0.05 a
T3=200 µM SNP	15.75±1.52	2.60± 0.26 a	1.06± 0.12 b	0 10± 0.03 a
T4=300 µM SNP	12.89±0.51	2.07± 0.25 ab	0.31± 0.04 c	0 04± 0.01 b
LSD p≤ 0.05	ns	0.22	0.10	0.02

Different letters denote significant difference between means (Least Significant Difference Test, P<0.05)

Number of roots (per plantlet)

The highest number of roots is in the T1 treatment, 15.94 (per plant), followed by the T2 treatment with an increasing number of roots, 15.39 (per plant). The lowest number of roots was 8.56 (per plant) in the T4 application (Table 1). The SNP is known to promote cell division and differentiation. With the differentiation of cells, primary root and capillary roots occur. SNP applications in our study increased the number of roots positively. However, as the increasing dose inhibited root formation, the number of roots decreased. Based on this idea, SNP application may vary depending on the plant type and dose. T1, T2 and T3 application doses affected the number of roots positively.

In a study on SNP applications, examining the root number in *Stevia rebaudiana* (Bertoni) under PEG-induced drought stress revealed that the highest root number was obtained in the control treatment, and an increasing concentration of SNP reduced the root number (Pradhan *et al.*, 2020). The results of our study are in agreement with Ötvos *et al*. (2005), who reported that NO may interact with auxins coupling the regulation of cell division and differentiation of plant cells.

Root length (cm)

Sodium nitroprusside, a nitric oxide donor, was used in the study. NO has recently been recognized as a new type of phytohormone (Leterrier *et al*., 2012). As a result of the treatments applied in the study, a statistically significant difference was observed when the plantlets were examined in terms of root length. When examining the plantlets in terms of root length as a result of the applied treatments in the study, a statistically significant difference was observed. The highest root length was 9.27 cm in the T3 application, while the lowest root length was 3.03 cm in the T4 application (Table 2; Figure 2) NO accumulation in roots mediates auxin induction of lateral root formation, adventitious root growth (Tewari *et al*., 2008), and root hair development. In addition to increased root formation (Lombardo *et al*. 2006), root length also increased in SNP treatments compared to the control treatment containing only auxin. The success of micropropagation under *in vitro* conditions is dependent on the number and length of roots, as well as the number of healthy surviving plants during the rooting and acclimatization stages (Diaz *et al*., 2010; Demir *et al*., 2015; Tesfa *et al*., 2016).

Some studies have utilized SNP to improve the adverse conditions caused by abiotic stress. In line with research on SNP use, our study aligns with findings such as the positive effect of exogenous SNP applications on root length in lentils under salinity stress, with 100 µM SNP application enhancing root length (Yasir *et al*., 2021), and the significant increase in root length in lettuce under salinity stress through foliar application of SNP (Sardar *et al*., 2023). As mentioned by Blazich and Heuser (1979), the acceleration of cell division in the root region by SNP and IAA leads to an increase in root length. In this context, our study's results are supported by other studies that have found positive effects of SNP on root length (Sarropoulou *et al*., 2014; Ragaey *et al*., 2022).

Number of leaves

Nitric oxide is a ubiquitous bioactive molecule involved in a wide array of plant developmental processes, including germination, flowering, fruit ripening, and organ senescence (Crawford and Guo, 2005). Recently, nitric oxide (NO) has been a focus of efforts to improve *in vitro* plant regeneration. SNP added to the medium under *in vitro* conditions positively affects plant development by increasing root formation and carrying water and nutrients from the culture medium to the upper parts of the plant through the roots. This positive effect played a role in the number and development of leaves. However, plants developed better in culture media containing SNPs compared to the control. In this study, there was no significant difference among the treatments in terms of leaf number. The highest leaf number was observed in the T3 (15.75) application, while the lowest leaf number was in the T4 (12.89) application (Table 2). Previous studies have found that SNP applications improve the adverse effects on plants under stress conditions and positively enhanced vegetative growth (Habib *et al*., 2020; Pradhan *et al*., 2020; Gougerdchi *et al*., 2021; Jafari and Shahsavar, 2022; Sardar *et al*., 2023). Similar studies have also shown that SNP increases leaf number, supporting our findings.

Shoot length (cm)

When examining the plantlets in terms of shoot length as a result of the applied treatments, a statistically significant difference was found. The highest shoot length was observed in the T3 (2.60) application, while the lowest shoot length was in the T1 (1.81) application (Table 2). Nitric oxide (NO) has been reported to regulate cell division and be effective in cytokinin signal transmission, thus enhancing the success of micropropagation (Ötvös *et al*., 2005). It is stated that SNP exhibits some cytokinin effects in plants (Scherer and Holk, 2000) and promotes cell division and elongation. In addition to promoting plant growth and development, SNP also mitigates the adverse effects of various biotic and abiotic stress conditions (Sundararajan *et al*., 2022). Studies in this field have shown that SNP and Putrescine applications alleviate the effects of drought stress on tomato seedlings and increase shoot length (Sundararajan *et al*., 2022). In another *in vitro* study, SNP application to Allium hirtifolium increased shoot regeneration in plants under drought stress condition (Ghassemi-Golezani *et al*., 2018). Similar studies supporting our findings have also demonstrated that SNP application increases shoot length (Ali *et al*., 2017; Jafari and Shahsavar, 2022). Since SNP has a direct effect on the cell wall, it has been reported that it can cause cell wall loosening, trigger cell expansion and stimulate plant growth (Lamattina *et al*. 2003). In this study, SNP increased shoot length compared to the control. The results in our study were similar to the reported effects of the SNP.

Plant fresh weight (g)

NO ensures the growth and development of the plant by enabling the cells to expand and elongate. It is thought that growing plants directly increases their biomass (Beligni and Lamattina, 2001). When evaluating the plantlets in terms of fresh weight as a result of the applied treatments, a statistically significant difference was found. The highest plant fresh weight was observed in the T1 (1.74 g) application, while the lowest was in the T4 (0.31 g) application (Table 2). SNP increases the plant's photosynthesis rate and chlorophyll content. The increased photosynthesis contributes to an increase in dry matter within the plant, leading to an increase in both fresh weight and dry weight (Per *et al*., 2017; Aras, 2018; Ekinci *et al*., 2018). Some studies have observed that applications of SNP, especially in combination, enhance plant resistance under adverse conditions and prevent a decrease in plant fresh weight (Mahendran *et al*., 2021; Jafari and Shahsavar, 2022; Zhang *et al*., 2023).

Plant dry weight (g)

There is a significant difference among the treatments in terms of plant dry weight. The highest dry weight was determined in the T1 (0.12 g) application, while the lowest dry weight was in the T4 (0.04 g) application (Table 2). In studies on the effects of SNP application in drought stress in Mexican lime, the highest dry weight was reported in the 25 µM SNP application, while the lowest dry weight was obtained from the 3% PEG application (Jafari and Shahsavar, 2022). In a study on alleviating the effects of temperature stress in wheat through SNP and GA_3 applications, it was reported that the combination of SNP and GA_3 significantly increased dry weight compared to the control (Zhang *et al*., 2023). In the study, different doses of SNP positively influenced plant dry weight, confirming previous findings.

Principal Component Analysis (PCA)

PCA is a statistical test related to factor analysis. PC is a linear combination of data sets resulting from the applications that preserves some correlations between them. In order to better understand the effects of SNP on vegetative growth, a PCA analysis was used to cluster similar parameters (Figure 3). It is displayed along two different principal components (PC) for all examined parameters.

While 71.7% of the variance is observed along PC[1], PC[2] represents 27.37% of the variation. Although the control treatment (T1) does not necessarily need to be directly related to any of the other applications, it was observed to be positioned on the positive side of PC. T2 and T3 applications were highly correlated with parameters P1, P2, P5, and P6 along the positive axis of PC2, separating these groups from the control and parameters P3, P4, P7, and P8. T4 application was distinctly separated from all other groups (Figure 3). Both PC[1] and PC[2] showed that the P2 parameter had the lowest score. However, it was observed that T2 and T3 applications formed one group, while T1 and T4 applications formed a different group. This indicated that T2 and T3 applications had positive effects on Aronia plantlets. The T4 application showed a formation that was distant from the control group and growth parameters. Additionally, the T4 application negatively affected growth parameters. Under stress conditions, plant development is interrupted, making it necessary to implement practices that can alleviate this situation. However, the concentration of chemicals applied may vary depending on the species, genotype, and variety. In our study, it was found that increasing SNP concentration limited the vegetative development of Aronia plantlets, and the T4 application was placed in a different group in the PCA analysis.

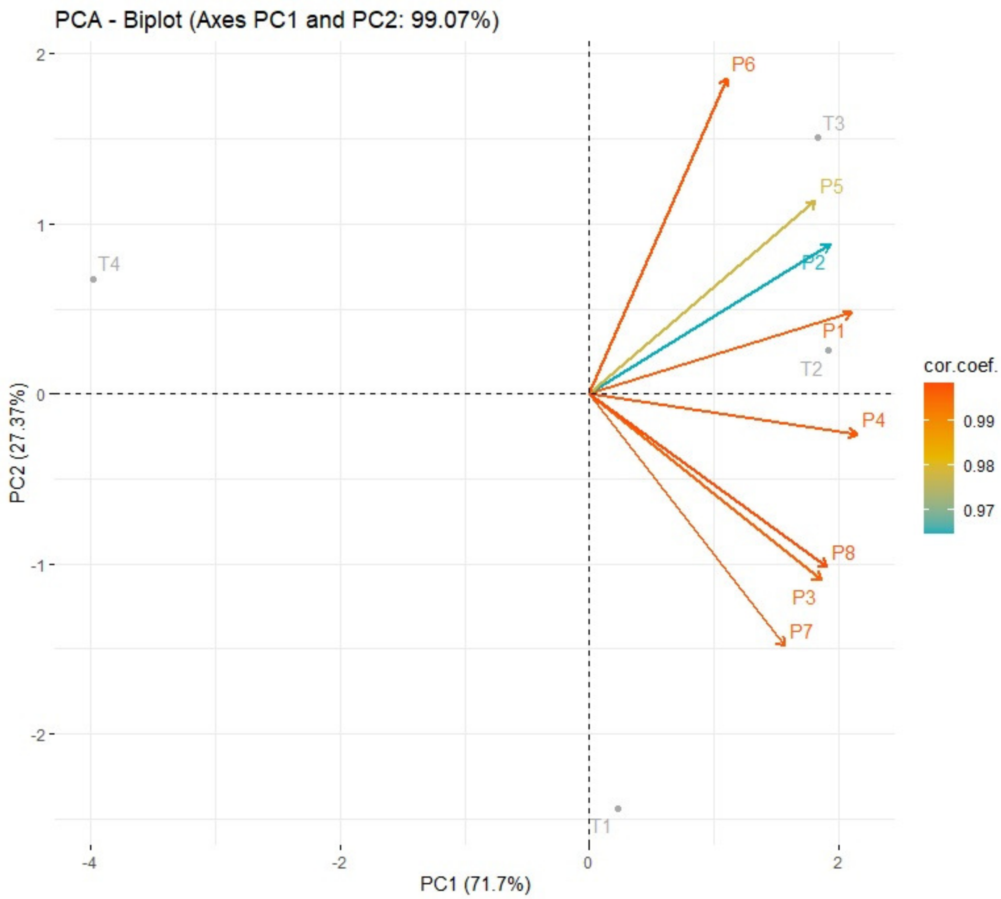

Figure 3. The loading plot of all identified variables included in PCA for morphological parameters in Aronia plantlets. P1: Survival rate (%), P2: Rooting rate (%), P3: Number of roots (pieces/plantlet), P4: Root length (cm), P5: Number of leaves (pieces), P6: Shoot length (cm), P7: Plant fresh weight (g), P8: Plant dry weight (g). SNP treatments: T1=0 µM SNP; T2=100 µM SNP; T3=200 µM SNP; T4=300 µM SN

Hierarchical Clustering Analysis (HCA)

Hierarchical Clustering Analysis (HCA) is a clustering method that examines samples in groups (Lee and Yang, 2009; Granato *et al*., 2018). The result of HCA is typically presented in a dendrogram, a graphical representation showing the relationships between samples. In this study, HCA was performed using 8 different parameters (Figure 4). The applications were clustered into I, II, and III (Figure 4).

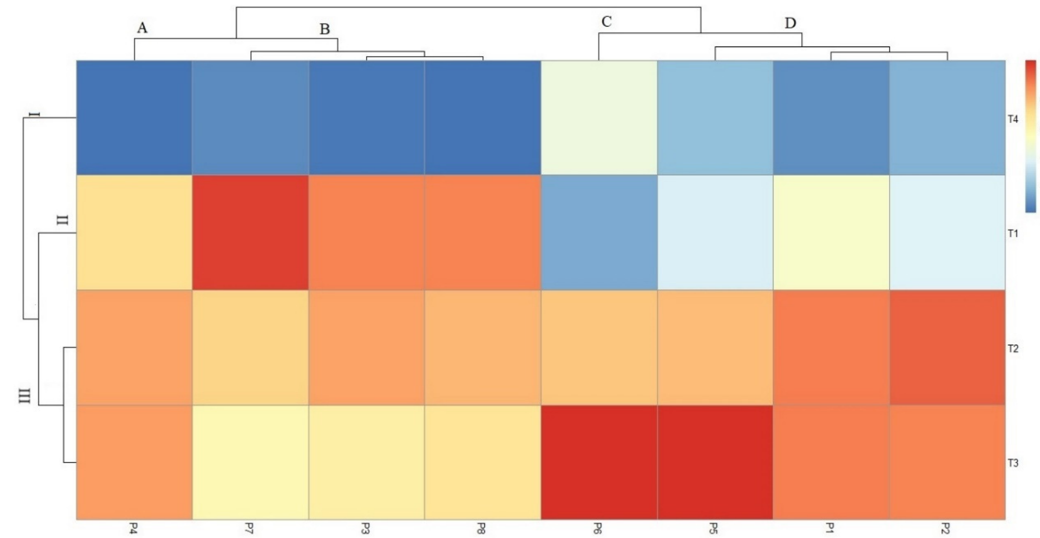

Figure 4. Heatmap for vegetative growth parameters evaluated under control and application conditions in *Aronia melanocarpa* [Michx.] Elliot. P1: Survival rate (%), P2: Rooting rate (%), P3: Number of roots (pieces/plantlet), P4: Root length (cm), P5: Number of leaves (pieces), P6: Shoot length (cm), P7: Plant fresh weight (g), P8: Plant dry weight (g). I, II, III Represent the application groups studied. A, B, C and D represent the parameter groups studied. SNP treatments: T1=0 µM SNP; T2=100 µM SNP; T3=200 µM SNP; T4=300 µM SNP

The parameters were grouped into four categories: A, B, C, and D. T4 treatment was placed in cluster I, T1 in cluster II, and T2 and T3 treatments in cluster III. In group A, parameters P4; in group B, parameters P3, P7, and P8; in group C, parameter P6; and in group D, parameters P1, P2, and P5 were included. The T3 treatment showed the highest positive increase in parameters P5 and P6. The T3 treatment corresponds to the 200 µM SNP dose. As a result of this treatment, the parameters of leaf number (P5) and shoot length (P6) showed a positive increase in growth parameters. A higher number of leaves and an extended shoot length facilitate the plant's adaptation to external conditions and increase the photosynthesis rate (Ghassemi-Golezani *et al*., 2018; Rasheed *et al*., 2022; Emamverdian *et al*., 2023). The T4 treatment in cluster I showed a negative trend in growth parameters (P1, P2, P3, P4, P5, P6, P7, and P8). The reason for this trend is that sodium nitroprusside, the nitric oxide donor used in the treatment, although improving the plant under stress conditions, becomes toxic as the concentration increases. Therefore, as the concentration increases, the development of growth parameters is negatively affected. Studies have reported that increasing concentrations of corrective chemicals have a toxic effect on plants (Trifunović-Momčilov *et al*., 2023).

Conclusions

Nitric oxide (NO) plays various roles in the growth and development of plants. In this study, the effects of sodium nitroprusside (SNP), a NO donor, on shoot proliferation and regeneration were investigated. The vegetative development of plants growing under stress conditions is significantly restricted. Based on this consideration, applications that positively affect vegetative development in such situations will reduce this pressure significantly. In addition, well-developed root structures facilitate plant adaptation during acclimatization under *in vitro* conditions and reduce plant loss. Our study in a well-developed root system was observed in the plantlets after sodium nitroprusside application. According to the study results, which positively affect vegetative development, were determined suitable doses. Additionally, the study revealed that high SNP concentration has a toxic effect. In sum, it is of note that SNP, a donor of NO, has a direct effect on *in vitro* root induction, shoot differentiation, and vegetative development of the *Aronia melanocarpa* L explants. The results reveal how important sodium nitroprusside, a nitric oxide donor, plays in cell division and differentiation in plants

Authors' Contributions

Investigation and conceptualization, BEA, NS, YA, ŞK, HE; methodology, NS, HE (Heydem Ekinci); software and formal analysis, NS, YA, HE (Heydem Ekinci); writing-original, writing-review and editing, and draft preparation, HE, BEA, YA, ŞK and NS; visualization HE (Heydem Ekinci). All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

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

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