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DEVELOPMENT OF AN EFFECTIVE STERILIZATION PROTOCOL FOR PLANT TISSUE CULTURE STUDIES IN SUPERFRUIT ARONIA [*Aronia melanocarpa* (Michaux) Elliot]

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Abstract: Effective sterilization protocols are crucial for a successful tissue culture study in *Aronia*. These protocols directly influence contamination rates, shoot health, and root development. In this context, the study aims is to develop an effective sterilization protocol for plant tissue culture studies in *Aronia* [*Aronia melanocarpa* (Michaux) Elliot], commonly known as the "superfruit." In the study, the Nero *Aronia* variety shoot tips were used as material. The sterilized shoot tips were transferred to the respective plant tissue culture media in a randomized parcels trial pattern with three replicates, each containing three explants per replicate. Various concentrations and combinations of sterilizing agents, such as sodium hypochlorite (NaOCl), hydrogen peroxide (H_2O_2), mercuric chloride ($HgCl_2$), and ethanol (C_2H_5OH), were evaluated to determine their effectiveness in maintaining tissue health and reducing contamination. Twelve protocols were developed, incorporating different concentrations of these chemicals. The data were subjected to statistical analysis using the SAS software package (LSD0.05-Proc GLM). Analysis of variance (ANOVA) was performed to evaluate differences among sterilization treatments for each parameter. The results showed that the combination of 5% NaOCl and 3% H_2O_2 (10 min each) provided the lowest average contamination rate 0.0%, the highest average number uncontaminated explants 9.0 pieces, shoot length 3.0 cm and root length 2.5 cm demonstrating the sterilization efficiency of this combination. On the other hand, protocols containing $HgCl_2$, especially at higher concentrations, resulted in impaired root development. High ethanol concentrations also contributed to effective sterilization, with the combination of 7% NaOCl (10 min) and 80% ethanol (5 min) yielding a low contamination rate (22.0%) and preserving tissue health. This study emphasizes balancing sterilization protocols between effective contamination control and tissue viability. The findings are expected to benefit the improvement and development of tissue culture techniques for *Aronia* and similar species, providing a basis for further research on effective sterilization practices, currently limited in *Aronia* tissue culture.

Keywords: Aronia [Aronia melanocarpa (Michaux) Elliot], in vitro, Sterilization, Contamination rate, Shoot length, Root length

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1. Introduction

Aronia melanocarpa, commonly known as chokeberry, has garnered significant attention in recent years due to its high antioxidant capacity and rich nutritional profile, making it valuable in agricultural and pharmaceutical sectors (Knudson, 2009; Sikora et al., 2014; Fidancı, 2015). Particularly rich in phenolic compounds, flavonoids, and anthocyanins, *Aronia* is renowned for its health benefits and holds great potential for use in functional foods, dietary supplements, and pharmaceuticals (Oszmiański and Wojdyło, 2005; Hannan, 2013; Jurikova et al., 2017). However, various biotic and abiotic stress factors limit this species large-scale production and propagation (Kulling and Rawel, 2008). The difficulty in propagating *Aronia* using conventional methods, especially when aiming to produce high-quality plant material, underscores the need for tissue culture techniques to overcome these challenges (Almokar and Pırlak, 2018).

Tissue culture is a biotechnological method based on the aseptic cultivation of plant cells, tissues, or organs in a controlled environment (George and Sherrington, 1984). This technique is particularly effective in clonal propagation and preserving plant genetic resources. However, one of the primary challenges in tissue culture systems is contamination, which arises during the sterilization of explants (Leifert and Cassells, 2001). Contamination is typically caused by bacteria, fungi, or viruses that enter the culture medium, hindering the healthy growth of plant tissues. As a result, contamination significantly lowers the success rate of tissue culture experiments and compromises the reliability of the results (Cassells, 2012). Consequently, the sterilization step is critical to the success of tissue culture procedures (Kaya et al., 2023).

Sterilization is usually achieved through various

chemicals that eliminate contaminants and allow the explants to grow in aseptic conditions (Kaya and Sarıyer, 2024). Sodium hypochlorite (NaOCl), hydrogen peroxide (H_2O_2), mercuric chloride ($HgCl_2$), and ethanol (C_2H_5OH) are among the most commonly used sterilizing agents in plant tissue culture. NaOCl, a powerful oxidizing agent, provides effective sterilization even at low concentrations, while H_2O_2 is often used in combination with NaOCl to enhance sterilization efficiency (Al Ghasheem et al., 2018). However, although agents like $HgCl_2$ are highly effective at reducing contamination, they can have toxic effects on plant tissues, particularly at higher concentrations (Cargnelutti et al., 2006). Ethanol, on the other hand, is known for its ability to provide effective surface sterilization while maintaining low toxicity, preserving the viability of the explants (Kampf and Kampf, 2018).

The application of various sterilization methods in tissue culture for *Aronia* plants is crucial for the healthy growth and propagation of the species (Mannino et al., 2021). Tissue culture is a technique that allows the multiplication of plants using cellular or tissue samples in a laboratory setting (Rai et al., 2022). In this process, it is essential to prevent contamination of the plant material or cells being used (Park, 2021). Sterilization helps eliminate pathogens and pests, facilitating the acquisition of healthy plant material (Fielder et al., 2024). The risk of contamination increases with the introduction of microorganisms and pathogens from the external environment into the laboratory (Singh et al., 2023). Therefore, sterilizing all materials, including seeds, tissue samples, and nutrient media, is a critical step (Rout and Jain, 2020). Moreover, the quality and yield of *Aronia* plants propagated through tissue culture are closely linked to the effectiveness of the sterilization processes. Sterilized materials support the development of healthy and resilient plants while minimizing losses caused by diseases and pests (Angel et al., 2021). The implementation of various sterilization methods in tissue culture for *Aronia* is essential for ensuring a successful production process and obtaining high-quality plants (Anuruddi et al., 2023). Because chokeberry fruit is rich in phenolic compounds such as anthocyanins, flavanols and phenolic acids, intense tissue darkening occurs during the initial phase of in vitro studies when explants are first cultured. Due to this tissue darkening, it is not possible to progress to the shoot multiplication phase of micropropagation studies. It will be possible to complete the in vitro shoot multiplication phase in a healthy manner by testing different sterilisation techniques to eliminate intense phenolic compounds and by identifying and using sterilisation techniques that can effectively eliminate tissue darkening.

The primary objective of this study is to optimize the sterilization protocol for *Aronia* species, thereby improving tissue culture efficiency. Specifically, the study aims to investigate the effects of various combinations of sterilizing agents on biological parameters such as contamination rate, shoot health and root development. By using different concentrations of NaOCl, H_2O_2, $HgCl_2$, and ethanol, this research seeks to identify protocols that maximize sterilization efficacy and minimize the toxic effects on plant tissues. In this context, the study contributes to the existing literature by offering practical solutions for tissue culture challenges in *Aronia* and other economically important plant species.

2. Materials and Methods

2.1. Plant Material

In the study, the Nero *Aronia* variety shoot tips were used as explant sources during the active growth period in May. Plant materials were taken from actively growing shoots, approximately 1-2 cm in length, from five-year-old healthy and disease-free plants grown under the ecological conditions of Çanakkale, Türkiye. The explant selection criteria focused on viability and the absence of visible damage or contamination. After collection, the explants were immediately transferred to the laboratory for sterilization and culture initiation.

2.2. Surface Sterilization of Explants

To determine the most effective sterilization protocol for *Aronia* explants, various combinations of sterilization agents were evaluated. These combinations included sodium hypochlorite (NaOCl), hydrogen peroxide (H_2O_2), mercuric chloride ($HgCl_2$), and ethanol (C_2H_5OH) at different concentrations (Table 1). The sterilization process was carried out in three main stages: pre-treatment, surface sterilization, and final rinsing. Pre-treatment: the explants were washed under running tap water for 30 minutes to remove surface dust and debris. They were then immersed in 1% (v/v) Tween-20 solution for 10 minutes to clean the surface further. Surface sterilization: the explants were subjected to various treatments according to the combinations and concentrations in Table 1. Each sterilization treatment involved soaking the explants in the respective sterilizing agent for a specified duration. Sequential rinsing with sterile distilled water was performed for protocols involving NaOCl, H_2O_2 and $HgCl_2$. Final rinsing: after surface sterilization, the explants were rinsed three times with sterile distilled water to remove chemical residues and minimize toxicity (Soylu and Erturk, 1999).

2.3. Culture Medium Preparation

Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) was used as the basal medium for the explants. The components were added to the medium: 30 g/L sucrose, 0.8% (w/v) agar, plant growth regulators: 2 mg/L 6-benzylaminopurine (BAP) for shoot multiplication and 1 mg/L indole-3-butyric acid (IBA) for root induction. The pH of the medium was adjusted to 5.8 using 0.1 N HCl and 0.1 N NaOH, and the medium was autoclaved at 121 °C for 20 minutes. The protocol stated by Şengül (2012) was followed while preparing the culture media. After cooling, the medium (approximately 15 ml) was poured into sterile 75x75x100 mm magenta boxes.

Table 1. Sterilization combinations and application durations determined within the scope of the study

Protocol No	Sterilization Combination	Duration (min)	Washing
1	%1 NaOCl	10	x3
2	%3 NaOCl + %70 C_2H_5OH	15 + 5	x3
3	%5 NaOCl + %3 H_2O_2	10 + 10	x3
4	%7 NaOCl + %80 C_2H_5OH	10 + 5	x3
5	%0.1 $HgCl_2$ + %70 C_2H_5OH	5 + 5	x3
6	%0.5 $HgCl_2$ + %80 C_2H_5OH	10 + 5	x3
7	%1 $HgCl_2$ + %3 H_2O_2	10 + 10	x3
8	%0.1 $HgCl_2$ + %5 NaOCl	5 + 10	x3
9	%5 H_2O_2 + %70 C_2H_5OH	15 + 5	x3
10	%3 H_2O_2 + %5 NaOCl	10 + 10	x3
11	%7 H_2O_2 + %70 C_2H_5OH	10 + 5	x3
12	Distilled water (Control)	5	x3

After planting, the magenta boxes were cultured for four weeks at 25±1 °C, under a 3000 lux fluorescent lamp with a 16- hour photoperiod.

2.4. Evaluated Parameters

The effectiveness of the sterilization treatments was assessed over a 4-week culture period, and the following parameters were recorded:

a. Contamination rate: The percentage of explants showing contamination for each treatment was calculated.
b. Number of healthy explants: The number of uncontaminated explants was recorded.
c. Shoot length: The average shoot length was measured in centimeters.
d. Root length: The average root length was measured in centimeters.

2.5. Statistical Analysis

The data were subjected to statistical analysis using the SAS software package (LSD0.05-Proc GLM). Analysis of variance (ANOVA) was performed to evaluate differences among sterilization treatments for each parameter. Results with a p-value less than 0.05 ($P<0.05$) were considered statistically significant (Karaokur et al. 2019).

3. Results

When examining the study findings, it was observed that explant samples were treated with 1% NaOCl for 10 minutes in the first treatment. The results showed an average contamination rate of 54.22%, an average number of healthy explants of 4.12, an average shoot length of 2.1 cm, and an average root length of 1.8 cm. In the second treatment, explant samples were treated with 3% NaOCl for 15 minutes and 70% ethanol for 5 minutes. The average contamination rate was 32.22%, the average number of healthy explants was 6.10, the average shoot length was 2.3 cm, and the average root length was 2.0 cm. In the third treatment, explant samples were treated with 5% NaOCl for 10 minutes and 3% H_2O_2 for 10 minutes. The average contamination rate was 0.0%, the average number of healthy explants was 9.00, the average shoot length was 3.0 cm, and the average root length was 2.5 cm. In the fourth treatment, explant samples were treated with 7% NaOCl for 10 minutes and 80% ethanol for 5 minutes. The results showed an average contamination rate of 22.0%, an average number of healthy explants of 7.02, an average shoot length of 2.9 cm, and an average root length of 2.3 cm. In the fifth treatment, explant samples were treated with 0.1% $HgCl_2$ for 5 minutes and 70% ethanol for 5 minutes. The average contamination rate was 75.55%, the average number of healthy explants was 2.20, the average shoot length was 1.9 cm, and the average root length was 1.5 cm. In the sixth treatment, explant samples were treated with 0.5% $HgCl_2$ for 10 minutes and 80% ethanol for 10 minutes. The average contamination rate was 65.22%, the average number of healthy explants was 3.13, the average shoot length was 2.0 cm, and the average root length was 1.7 cm. In the seventh treatment, explant samples were treated with 1% $HgCl_2$ for 10 minutes and 3% H_2O_2 for 10 minutes. The results showed an average contamination rate of 41.11%, an average number of healthy explants of 5.30, an average shoot length of 2.5 cm, and an average root length of 2.1 cm. In the eighth treatment, explant samples were treated with 0.1% $HgCl_2$ for 5 minutes and 5% NaOCl for 10 minutes. The average contamination rate was 53.0%, the average number of healthy explants was 4.23, the average shoot length was 2.2 cm, and the average root length was 2.0 cm. In the ninth treatment, explant samples were treated with 5% H_2O_2 for 15 minutes and 70% ethanol for 5 minutes. The average contamination rate was 9.88%, the average number of healthy explants was 8.11, the average shoot length was 2.0 cm, and the average root length was 2.4 cm. In the tenth treatment, explant samples were treated with 3% H_2O_2 for 10 minutes and 5% NaOCl for 10 minutes. The results showed an average contamination rate of 19.88%, an average number of healthy explants of 7.21, an average shoot length of 3.1 cm, and an average root length of 2.6 cm. In the eleventh treatment, explant samples were treated with 7% H_2O_2 for 10 minutes and 70% ethanol for 5 minutes. The average contamination rate was 7.55%, the average number of healthy explants was 8.32, the average shoot length was 2.9 cm, and the average root length was 2.5 cm.

Table 2. Effects of different sterilization method combinations on average contamination rate, number of healthy explants, shoot length and root length

Protocol No	Sterilization Combination	Duration (min)	Washing	Contamination Rate (%)	Number of Healthy (uncontaminated) Explants (pieces)	Shoot Length (cm)	Root Length (cm)
1	%1 NaOCl	10	x3	54.22^{d}	4.12^{e}	2.1^{bc}	1.8^{b}
2	%3 NaOCl + %70 C_2H_5OH	15 + 5	x3	32.22^{f}	6.10^{cd}	2.3^{bc}	2.0^{ab}
3	%5 NaOCl + %3 H_2O_2	10 + 10	x3	0.00^{i}	9.00^{a}	3.0^{a}	2.5^{a}
4	%7 NaOCl + %80 C_2H_5OH	10 + 5	x3	22.00^{g}	7.02^{c}	2.9^{a}	2.3^{ab}
5	%0.1 $HgCl_2$ + %70 C_2H_5OH	5 + 5	x3	75.55^{b}	2.20^{g}	1.9^{bc}	1.5^{ab}
6	%0.5 $HgCl_2$ + %80 C_2H_5OH	10 + 5	x3	65.22^{c}	3.13^{f}	2.0^{bc}	1.7^{bc}
7	%1 $HgCl_2$ + %3 H_2O_2	10 + 10	x3	41.11^{e}	5.30^{d}	2.5^{b}	2.1^{ab}
8	%0.1 $HgCl_2$ + %5 NaOCl	5 + 10	x3	53.00^{d}	4.23^{e}	2.2^{bc}	2.0^{ab}
9	%5 H_2O_2 + %70 C_2H_5OH	15 + 5	x3	9.88^{h}	8.11^{b}	2.0^{bc}	2.4^{a}
10	%3 H_2O_2 + %5 NaOCl	10 + 10	x3	19.88^{gh}	7.21^{bc}	3.1^{a}	2.6^{a}
11	%7 H_2O_2 + %70 C_2H_5OH	10 + 5	x3	7.55^{hi}	8.32^{b}	2.9^{a}	2.5^{a}
12	Distilled water (Control)	5	x3	100^{a}	0.00^{h}	1.5^{c}	1.2^{c}

The data reveals statistically significant differences among the values indicated with different letters ($LSD_{0.05}$).

In the twelfth treatment, the control group, explant samples were washed with water only. This resulted in an average contamination rate of 100%, an average number of healthy explants of 0.00, an average shoot length of 1.5 cm, and an average root length of 1.2 cm (Table 2).

4. Discussion

This study provides a foundation for selecting effective sterilization protocols for *Aronia* tissue culture. Future research should explore similar protocols for different plant species and tissue culture conditions. Additionally, long-term studies on tissue development and phytotoxicity will be beneficial for optimizing sterilization protocols.

Sterilization is a fundamental step in tissue culture to prevent microbial contamination, which can compromise plant development. Existing literature supports our findings that effective sterilization, such as the 5% NaOCl and 3% H_2O_2 combination, reduces contamination and promotes healthy explant and root growth. Bidabadi and Jain (2020) demonstrated that inadequate sterilization techniques often lead to increased contamination and reduced plant viability, emphasizing the necessity of effective disinfection protocols. NaOCl has long been recognized as an effective sterilizing agent in plant tissue culture. However, its concentration must be carefully optimized to avoid phytotoxicity. Like our findings, da Silva et al. (2016) demonstrated that while lower NaOCl concentrations could result in higher contamination, excessively high concentrations may lead to tissue damage. Therefore, balancing sterilant concentration with exposure time is crucial for tissue health. H_2O_2's role in enhancing sterilization efficiency by producing reactive oxygen species is well-documented. The synergy between NaOCl and H_2O_2 in our study reflects findings by Şen (2012), who demonstrated that H_2O_2 not only effectively sterilizes but also helps in breaking dormancy and improving explant viability in tissue culture. The oxidative stress induced by H_2O_2 is crucial in killing many microorganisms.

Ethanol is commonly used with other sterilants, primarily due to its ability to denature proteins and dissolve lipids, effectively killing a broad spectrum of microorganisms. Mercuric chloride ($HgCl_2$) is known for its strong antimicrobial activity and phytotoxic effects. In our study, high concentrations of $HgCl_2$ were poor root development. These results are supported by Mekonnen et al. (2013), who observed that while $HgCl_2$ effectively reduces contamination, it also leads to oxidative damage and cellular toxicity in plant tissues, requiring careful handling and concentration adjustments. NaOCl is widely utilized across various plant species for its versatility as a sterilizing agent. For instance, Lema-Rumińska et al.

(2023) found that NaOCl was particularly effective for in vitro cultures of ornamental plants, which parallels our findings with *Aronia*. However, they also noted that different plant species exhibit varying sensitivity to NaOCl, underscoring the importance of species-specific sterilization protocols. Given $HgCl_2$'s toxic nature, there is ongoing research into finding safer alternatives. Studies by Vinh et al. (2024) have explored other sterilants, such as calcium hypochlorite and silver nanoparticles, as less toxic options. These alternatives offer reduced toxicity while still providing effective sterilization, making them promising candidates for future tissue culture protocols.

Explant viability is a critical factor influenced by sterilization and subsequent plant development. Ahmadpoor et al. (2022) demonstrated that sterilization protocols directly impact explant survival, with certain chemicals promoting or hindering growth depending on concentration and exposure time. Similar to our findings, their research stressed that an optimal sterilization balance promotes both explant survival and robust root development.

While traditional sterilization methods such as NaOCl, ethanol, and $HgCl_2$ are effective, a growing body of research focuses on biological and non-toxic alternatives, including plant-based antimicrobial compounds. Soumare et al. (2021) explored using essential oils as sterilizing agents in tissue culture, offering a natural alternative that minimizes tissue damage and contamination rates. These alternatives hold the potential for sustainable tissue culture practices.

Ethanol, is a potent sterilizing agent that may also play a role in tissue dehydration and hardening, leading to improved explant survival in some cases. Misra and Misra (2012) found that ethanol treatments could enhance tissue resilience against microbial attacks while simultaneously promoting shoot elongation, particularly in species with high moisture content, such as *Aronia*.

The long-term effects of sterilization methods on plant growth and development are important for future research. El-Sherif (2019) suggested that while certain protocols may be effective in the short term, they can have lasting impacts on genetic expression and tissue vigor. Longitudinal studies would provide deeper insights into how different sterilization methods influence tissue culture sustainability.

Our findings on the efficacy of NaOCl and H_2O_2 combinations in *Aronia* can be extended to other plant species. For example, Dagne et al. (2023) reported similar results in grapevine tissue cultures, showing that these sterilization methods are effective in reducing contamination and promoting healthy tissue growth across various plant genera. It is also important to note that environmental factors, such as humidity and light exposure during the sterilization process, can influence contamination rates and tissue health. Agrawal et al. (2024) demonstrated that sterile work conditions, in conjunction with optimal environmental factors, improve sterilization outcomes, suggesting that protocol adjustments may be necessary based on laboratory conditions. Our findings have practical implications for commercial tissue culture operations, particularly in ensuring the consistent production of healthy plants.

5. Conclusion

This study aimed to optimize sterilization protocols for *Aronia melanocarpa* explants in tissue culture. Various combinations of sterilizing agents at different concentrations were evaluated based on several biological parameters, including contamination rate, number of healthy explants, shoot length, and root length. The results demonstrate that the proper combination and concentration of sterilizing agents can effectively prevent contamination while promoting healthy explants growth and development.

The findings indicate that the combination of 5% NaOCl + 3% H_2O_2 was particularly effective in reducing contamination while supporting a high rate of number of healthy explants. This combination yielded the lowest contamination rate at 0.0% and resulted in healthy growth in 9.00 explants. Additionally, it was observed that higher concentrations of $HgCl_2$ exhibited toxic effects on explants, negatively impacting both shoot length and rooting rates. The use of high-concentration ethanol, such as 70% C_2H_5OH, provided effective surface sterilization while maintaining explant health due to its low toxicity.

This study highlights the critical importance of carefully optimizing the concentrations of sterilization agents to enhance tissue culture success in sensitive plant species like *Aronia*. The prevention of contamination, combined with the mitigation of toxic effects on explants, indicates that lower concentrations of agents with longer exposure times are more effective. The results underscore the increased efficiency of combined NaOCl and H_2O_2 treatments in improving sterilization outcomes.

Future studies could further refine sterilization protocols through broader testing on different *Aronia* species and other stone fruit types. Additionally, long-term evaluations of sterilization agents' effects at the culture initiation stage and throughout the entire growth and development process would provide a more comprehensive understanding of tissue culture success. Exploring alternative sterilization agents, such as biologically based methods, and investigating new sterilization techniques with lower toxicity could be considered in future research. Ultimately, this study provides a solid foundation for integrating *Aronia* tissue culture protocols into broader agricultural and biotechnological applications and opens new avenues for future research.

Author Contributions

The percentage of the author(s) contributions is presented below. All authors reviewed and approved the final version of the manuscript.

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C	80	20
D	60	40
S	70	30
DCP	40	60
DAI	50	50
L	60	40
W	60	40
CR	50	50
SR	90	10
PM	70	30
FA	50	50

C=Concept, D= design, S= supervision, DCP= data collection and/or processing, DAI= data analysis and/or interpretation, L= literature search, W= writing, CR= critical review, SR= submission and revision, PM= project management, FA= funding acquisition.

Conflict of Interest

The authors declared that there is no conflict of interest.

Ethical Consideration

Ethics committee approval was not required for this study because of there was no study on animals or humans.

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