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THE USABILITY OF EXTRACTS OF WALNUT GREEN OUTER SHELL FOR IN VITRO SURFACE STERILIZATION OF ROSEHIP (*Rosa canina* L.) PLANT

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Abstract: In this study, it was aimed to determine the efficiency ratios of extracts obtained from walnut outer green shell at different concentrations in order to provide surface sterilization in in vitro propagation of rosehip plant. Axillary buds taken in June were used as explants. After surface sterilization of the explants with walnut outer shell extract, they were cultured in MS0 nutrient medium. The lowest contamination rate on the 5th and 7th days was observed in the control and 20K groups. In addition, the highest number of shoots per explant was obtained in the 20K group. Uncontaminated explants were subcultured to MS0 medium containing 1.0 mg /L IBA + 1.0 mg /L BAP and 1.0 mg/L BAP + 1.0 mg/L NAA at the end of the 7th day. Rooting was not observed in the explants due to browning. In the in vitro propagation of plants, extract (20K) obtained from oven-dried walnut shell can be used instead of chemical sterilant.

Keywords: *Rosa canina* L., Walnut shell extract, Tissue culture, Antimicrobial activity, Antifungal activity

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1. Introduction

Rosa canina L. (Rosaceae) is a wild-growing shrub that is used not only for its nutritive and therapeutic properties (Wegg and Townsley, 1983; Kazaz et al., 2009) but also as a rootstock for garden roses (Khosh-Khui and Sink, 1982; Balaj and Zogaj, 2011). *Rosa canina L.*can be propagated by seeds although the germination percentage is low, but vegetative propagation is a better method to preserve the desired traits and propagate selected wild genotypes. (Pawłowski et al., 2020). Many types of roses are difficult to root, traditional propagation methods are too slow, time consuming and tiring. Tissue culture is becoming traditional more important as an alternative to plant propagation methods (Roberts and Schum, 2003). Compared to traditional plant propagation methods, micropropagation has advantages such as propagation of elite clones, production of large numbers of plants from the selected genotype, production of pathogen-free plants, and propagation of plants throughout the year (Kavand et al., 2011; Pahnekolayi et al., 2014). Although clonal micropropagation of roses is common, the developed protocols are not universally applicable to all species within the *Rosa L*. genus due to high heterozygosity and polyploidy. Consequently, separate micropropagation techniques need to be developed for each genotype (Bhat, 1992; Pati et al., 2006; Canli and Kazaz, 2009; Shirdel et al., 2013).

In vitro protocols offer a way to shorten growth cycles, and plant propagation from axillary buds has proven to be a widely applicable method that ensures high growth rates and maintains clonal fidelity (Ngezahayo and Liu, 2014). The initial stage of micropropagation is influenced by various parameters, and it has been found that buds from softwood trunks are more sensitive than those from hardwood (Mederos and Enriquez, 1987). Significant differences in shoot proliferation rates have been observed in different *R. hybrida* cultivars, depending on the position of the node on the stem (Bressan et al., 1982). The performance of nodal segments is much better than the shoot tips (Horn, 1992). Cultivation of *Rosa canina L*. by non-traditional techniques offers many advantages resulting from its opportunities to improve, protect and maintain a plant of food and pharmaceutical value (Van der Mark et al., 1990). It is critical to prevent microbial contamination of plant tissue cultures or for successful micropropagation. Epiphytic and endophytic organisms can cause severe losses in micropropagated plants at all stages of growth (Cassells, 1991). The expected contamination rate is generally very low when using young plants or seedlings grown in a greenhouse. However, during the propagation of selected wild genotypes, the contamination rate is expected to increase as well as the presence of pathogens, and care must be taken during the sterilization phase (Marković et al.,

2021). Causes of contamination in plant tissue cultures include acute contamination caused by ineffective superficial sterilization and contamination caused by microorganisms hidden in the explant or by microorganisms established during subculture. In addition, chronic contamination can occur naturally after a long sterile culture period (Babaoğlu et al., 2002). Walnut shell were found 13 different phenolic compounds such as juglone, chlorogenic acid, caffeic acid and gallic acid. The phenolic compound is found in all parts of the juglone walnut and is known for its antimicrobial effect (Stampar et al., 2006). It is also one of the oldest examples of allelopathy with its inhibitory effect on walnut plant species. The chemical responsible for walnut allelopathy is juglone (5-hydroxy-1,4 naphthoquinone) and its inhibitory effect on plant species is one of the earliest examples of allelopathy (Davis, 1928; Rice, 1984). Juglone has been isolated from various plants within the walnut family (*Juglandaceae*), including *J. nigra* and *J. regia* (Daglish, 1950; Prataviera et al., 1983). Walnut leaf extracts are used in allelopathic research due to their juglone content and antimicrobial activity (Clark et al., 1990, Dama et al., 1998, Tan et al., 2012). The antioxidant potential of the aqueous extracts of 6 walnut cultivars grown in Portugal (Franquette, Lara, Marbot, Mayette, Mellanaise and Parisienne) was determined. It was determined that they have a concentration-dependent antioxidant capacity, the lowest in the Parisienne variety (Pereira et al., 2008). In addition, antimicrobial capacities against gram positive *(Bacillus cereus, Bacillus subtilis, Staphylococcus aureus*) and gram negative bacteria (*Pseudomonas aeruginosa, Escherichia coli, Klebsiella pneumoniae*) and fungi (*Candida albicans, Cryptococcus neoformans*) were determined. In addition to the antimicrobial activities of all extracts, it was revealed that the effect of each walnut variety extract was different according to the tested microorganism (Pereira et al., 2008). In our study aims to determine the antimicrobial and antifungal efficacy of the walnut's green outer shell extract against contaminants that develop in tissue culture media, as well as to evaluate its regeneration capability. While there are numerous studies assessing the antimicrobial and antifungal effects of the walnut's outer green shell, this study holds significance as it pioneers the use of natural sterilization materials in in vitro studies, thereby minimizing chemical usage.

2. Materials and Methods

2.1. Plant Material

Axillary bud explants of the rosehip plant (*Rosa canina L.*) were utilized for in vitro regeneration in our study. The explants were collected from plants growing in nature in June 2022. Walnut outer green shells were acquired during the 2021 harvest. Some of these shells were subjected to drying in an oven at 45°C, while others were sun-dried. Oven-dried walnut green outer shells were weighed 10 g (10K), 15 g (15K) and 20 g (20K) and put into separate containers. Sun-dried walnut green outer shells were weighed 10 g (10Y), 15 g (15Y) and 20 g (20Y) and put into separate containers. The samples was added 50 ml ethanol. The extracts were subjected to a 3-hour hot water bath at 60°C, and the particulates were subsequently filtered out using Whatman No. 4 filter paper. The ethanol used as the solvent was removed using a rotary evaporator, and the crude extracts were obtained.

2.2. Sterilization

Two different surface sterilization methods were applied. Control group surface sterilization method; All explants were washed under tap water and then treated with 70% ethanol within a sterile cabinet for 5 minutes. For surface sterilization of explants they were treated with a 3% NaOCl (sodium hypochlorite) solution for 10 minutes, and then the explants (15 explants) were rinsed three times with bi-distilled water. These explants, with excess water removed using sterile blotting paper, were cultured in MS0 medium with one explant per test tube (Figure 1).

Figure 1. Explant sterilization with walnut outer green shell extract and control group.

Herbal surface sterilization method; All explants were washed under tap water and then treated with 70 % ethanol within a sterile cabinet for 5 minutes. For surface sterilization of explants, 10µl of each of the solvents named 10K, 15K, 20K and 10Y, 15Y, 20Y was added separately to 100 ml of bidistilled water. Sterilization mixture at 6 different concentrations was obtained. 15 explants in the sterilization mixture of each concentration were sterilized at 10 minutes. Excess water on the explants was removed using sterile filter paper and the explants were then transferred in MS0 medium with one explant in each test tube.

2.3. Preparation of MS0 Culture Medium

1000 ml bidistilled water were added 4.4 g of MS (PhytoTech, M519), 6.5 g agar (Condalab), 30 g sucrose (Merc) and its pH was adjusted to 5.7. This mixture was sterilized in an autoclave at 121 °C for 21 minutes. Then, this mixture mixture was then divided into tubes, 30 ml for each tube.

2.4. Culture of Explants

Each of the explants, which were previously sterilized in mixtures of different concentrations were transferred to MS0 culture medium in tubes.

2.5. Culture Conditions and Analysis

The explants were maintained in a climate cabinet at 23±2 °C with a light intensity of 3500 lux under a 16-hour light and 8-hour dark photoperiod. Contamination rates of the explants were assessed on the 2th, 5th, and 7th days in all treatment groups. At the conclusion of the 7th day, explants from all treatments were subcultured into MS medium containing 1.0 mg/L BAP + 1.0 mg/L IBA and 1.0 mg/L BAP + 1.0 mg/L NAA. The obtained results were subjected to analysis of variance in the Minitab 12.0 program. The determination of differences between the means was used to Tukey's multiple comparison test (Genç and Soysal, 2018).

3. Results and Discussion

The contamination rate and the number of shoots per explant by sterilization methods are given in Table 1. In all treatments, no contamination was observed in the explants on the 2th day of culture. On the 5th and 7th days, the best results were in the 20K and control groups ($P<0.01$). The best results in terms of the number of shoots per explant were determined in the 20K group. Compared to the control group, it can be said that walnut outer green shell extracts, which are prepared by drying in the sun and in an oven, result in significant sterilization during the sterilization phase. Browning was observed in subcultured explants and rooting was not achieved.

It is thought that different contamination agents developed in the culture medium and contamination that may occur during culturing affect this rate. Explants without contamination at the end of the 7th day were subcultured to MS medium containing 1.0 mg/L IBA + 1.0 mg/L BAP and 1.0 mg/L NAA + 1.0 mg/L BAP. Browning was observed in subcultured explants and rooting was not achieved. According to Vijaya et al. (1991), BAP was reported as the most effective growth regulator in stimulating shoot proliferation. Due to the browning and contamination of the explants, an evaluation could not be made in terms of the effectiveness of the two different auxins used in our study. Zapata et al. (1999), reported that meristem have inductive properties, so it is not necessary to use growth regulators in the shoot medium. The shoot numbers per explant indicated in our study were obtained in MS0 medium. Ambros et al. (2016), in their study of *Rosa canina L*. axillary buds, in contrast to the treatment of $AgNO_3$ with $HgCl_2$ approximately 98.0% of axillary explants reported that not contamination and the explants retained 59.6% of viability. In our study, the number of shoots per explant caused a decrease in many treatment groups due to the increase in contamination. Also, Ambros et al. (2016), reported that *Rosa canina L*. axillary buds exhibited low viability due to a high level of browning in axillary meristems collected in April-May, and explants isolated from leaf axils in July-August did not undergo development. They reported that the best results were obtained in the late vegetation period in September-October. It can be said that the reason for the low number of shoots per explant obtained in our study is due to the fact that the explants were obtained from June.

Table 1. Percentage contamination rates and number of shoots per explant on the 2th, 5th and 7th days of explants in the treatment carried out in June

Treatments	2nd	5th	7th	Number of shoots per explant
10 Y	0	21.97b	32.58b	0,57b
10K	0	21.97b	32.58b	0,50c
15 Y	0	40.98d	68.44c	0,14d
15K	0	21.97b	21.97a	0,43c
20Y	0	32.58c	40.98b	0,10d
20K	0	0.00a	21.97a	0,86a
Control	0	0.00a	21.93a	0,43c

The difference between treatment in the same column is significant ($P<0.01$).

Khorrami et al. (2018), investigated the potential of walnut green shell in the production of silver nanoparticles. AgNPs have been reported to exhibit antibacterial activity against standard strains of both Gram-positive and Gram-negative bacteria. In our study, sterilization was treatment with walnut outer green shell extracts for surface sterilization in plant tissue culture. As a result, it has been observed that sun-dried walnut outer shell solvent and oven-dried walnut outer shell solvent can be applied as a sterilization protocol.

Adebomojo and AbdulRahaman (2020), used biosynthesized nanosilver for the surface sterilization of Ocimum seeds and tissues in their study and evaluated its effects on callus formation. They concluded that there was no adverse effect on explant viability and callus formation, and that it could be used as an antimicrobial agent in surface disinfection, thus expanding the limits of potential treatmentof biosynthesized nanosilver in tissue culture. In our study, surface sterilization was achieved with walnut outer green shell extracts in order to ensure surface sterilization in plant tissue culture.

At the same time, no negative effects on explant viability were observed. Kocaçalışkan and Terzi (2001), investigated the allelopathic effects of walnut (*Juglans regia L*.) juglone and leaf extracts on seed germination and seedling. Juglone and different proportions of diluted

and undiluted walnut leaf extract were used. They reported that there was a positive correlation between the effects of juglone and the extracts. Seed germination was less affected than root and shoot development in all species. Cosmulescu et al. (2011), determined the amount of juglone in the leaves and green shell of five walnut varieties. It was observed that juglone was dominant in the green shell. They reported that the outer green shell and leaves of the walnut represent the most important source of walnut phenolics. In our study, no negative effects of extracts on shoot regeneration were observed. In addition, it was observed that the contamination rate was not high in the explants in the treatment group called 20 K and the number of shoots per explant was higher. The number of shoots per explant increased and decreased as a result of contamination and browning.

4. Conclusion

Contamination is a major problem in in vitro propagation of plants. Because contamination adversely affects the development of the plant in the culture medium and causes its death. The prevent contamination in the culture medium is used to more chemical agents. These chemical agents in some cases may adversely affect the development of the plant in the culture medium. In order to minimize these negative effects are researches carried out for the determination of herbal-based sterilization agents. In this study, walnut green outer shell was used as a sterilization agent in the in vitro propagation of the rosehip plant. The results revealed that walnut green outer shell can be used instead of chemical agents in sterilization.

Author Contributions

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

	E.Ş.	B.A.	A.K.
C	70	20	10
D	80	10	10
S	30	40	30
DCP	90	10	
DAI	100		
L	80	20	
W	80	10	10
CR	80	10	10
SR	80	10	10
PM	70	20	10
FA	80	10	10

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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