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Determination of optimal plant growth regulators for breaking seed dormancy and micropropagation of *Sorbus aucuparia* L.

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Abstract

Sorbus aucuparia L. is an important forest tree used in the reforestation of high-altitude mountainous areas, which carries significant pharmaceutical, industrial, ornamental, food, and ecological properties. However, the seed propagation and micropropagation of mature trees of *S. aucuparia* L., presents various difficulties which are integral aspects for the re-propagation and breeding of novel cultivars. In this study, we isolated the mature embryo-containing seeds of selected varieties of *S. aucuparia* L. from their seed coat and used them directly as explants *in vitro* to investigate and determine the optimal dose of cytokinin in breaking seed dormancy and micropropagation. 3 mg/L of benzyl adenine (BA) in addition to different concentrations and combinations of kinetin (0.5, 1, and 2 mg/L), indole-3-butyric acid (IBA) (0, 1, 0.5, and 1 mg/L), and 1-naphthalene acetic acid (NAA) (0, 1, 0.5, and 1 mg/L) were applied within a Murashige and Skoog (1962) (MS) medium at germination inhibition, shoot elongation, and shoot proliferation. In the MS mediums containing BA and kinetin, germination was achieved at the end of 1 week, and shoot proliferation was achieved at the end of 3 weeks. The most successful germination (96%), tallest shoot length (mean 5.1 cm), most shoot proliferation (mean 7.2 pieces), and number of nodes (mean 9.7 pieces) were identified in the MS containing 3 mg/L BA and 1 mg/L kinetin. Direct root formation with shoot elongation occurred in 25% of explants which germinated in the MS medium. For shoots propagated without roots in this medium, 62% of these achieved rooting at the highest dose of 1 mg/L indole-3-acetic acid (IAA) using a two-stage rooting method. Rooted shoots were successfully transferred to an *ex vitro* medium. These results provide a basis for breaking seed dormancy of selected *Sorbus* L. genotypes quicker, leading to more effective clonal production.

Keywords: *Sorbus aucuparia* L., rowanberry, tissue culture, seed dormancy, micropropagation, *in vitro*

Introduction

Sorbus aucuparia L., rowanberry, or mountain ash, is an important forest tree that grows slowly, which is used in large numbers for the reforestation of mountainous regions as well as for medical (Termentzi et al. 2008), industrial (Gabrelian 1972, Pamay 1994, Chalupa 2002), and ornamental (Lall et al. 2006, Suhonen et al. 2015, Jokimäki et al. 2017, Suhonen et al. 2017) purposes (Emam and Ghamarizare 2013). *S. aucuparia* L. is a durable forest tree that grows in the mountainous regions of Europe, from the plains up to the upper tree lines with heights between 10 m to 20 m. It can grow under harsh climate conditions on high mountains with humid soil, as well as in dry soil (Chalupa 2002).

In addition to being an important food source for birds and game animals in mountainous regions (Chalupa 2002, Suhonen et al. 2015, Suhonen et al. 2017), *S. aucuparia* L. is also widely used in the food industry (Gil-Izquierdo and Mellenthin 2001, Chalupa 2002, Baltacıoğlu 2006, Hukkanen et al. 2006, Vyviurska et al. 2015, Savikin et al. 2017, Bozhüyük et al. 2020).

Although *S. aucuparia* L. has significant potential uses in many areas and spreads naturally in forests including in Turkey, the *Sorbus* species has various problems in generative and vegetative production and is currently insufficient for meeting such needs. Seed production is very important in the propagation of the *Sorbus* species and in the production of novel cultivars (Grime et al. 1988). Seeds of the *Sorbus* species usually germinate after a few months in natural conditions, carrying a low survival rate for seedlings (Devillez et al. 1980) as the seeds of *Sorbus* L. species suffer from germination-related problems such as high embryo dormancy (Lenartowicz 1988). *Sorbus aucuparia* seeds are characterized by deep and abso-

lute dormancy with two components: embryo dormancy and seed coat dormancy (Devillez 1979a, Devillez et al. 1980, Raspé et al. 2000). When the fruits of these species are not eaten by animals, dormancy must be broken under natural conditions (Raspé et al. 2000, Paulsen et al. 2002). Breaking dormancy involves complex processes related to water intake and circulation, growth regulators, and trophic relationships (Devillez et al. 1980).

Sorbus seeds usually require between 9 to 26 weeks of cold stratification to break dormancy, whereby 6 months of cold stratification provides the highest germination rates for *S. acuparia* seeds (Devillez 1979a). Paganova (2007) and Var et al. (2010) reported that seeds of trees should be stratified for longer than 8 weeks at low temperatures and with high humidity to germinate, whereas Piagnani and Bassi (2000) reported that a 3-to-9-month period of stratification in a cold and moist environment is required to break seed dormancy of the *Sorbus* L. species. In another study, Drvodelic et al. (2018) analyzed the effects of *S. domestica* L. fruit size on seed germination and reported the highest germination rate (83.7%) in the seeds of large fruits (16 to 20 g) after 120-days of stratification. Whilst implementing 150 days of cold stratification for seeds, Tang et al. (2019) reported that cold stratification was efficient in the propagation of *S. alnifolia* seeds, which has a deep physiological dormancy. Similarly, Chalupa (2002) stated that the main method of production of *S. acuparia* is by seeds, which carries long stratification periods. Barclay and Crawford (1984) stated that the altitude from which *S. acuparia* seeds were taken from affects the required stratification time, where they reported that seedlings (and less than 10% germination) occurred in only the three lowest altitude samples (mean 8, 102 and 402 m) during an 18-week stratification period. Barclay and Crawford (1984) also noted that the altitude of the seed source affects the length of cold stratification required to break dormancy, where seeds from lower altitudes required longer treatments. Devillez (1979b) stated that embryo dormancy achieved a high germination rate after 16 ± 45 days of warm stratification (at 30°C or 30 ± 10°C, 12 hours-12 hours) followed by 6 months of cold stratification. Afroze and O'Reilly (2013) reported that only cooling was required to break dormancy in rowanberry seeds, but warm stratification reduced early germination and the amount of cooling required.

Vegetative propagation has been used to protect the superior characteristics of selected trees (Chalupa 2002). However, traditional vegetative propagation methods are not effective against the mature trees of *S. aucuparia* (Hansen 1990, Morabito et al. 1994, Chalupa 2002, Emam and Ghamarizare 2013). As a result, there is a need for more effective methods for the clonal propagation of plants selected from the natural environment, especially those which have several potential uses such as *S. aucuparia* (Chalupa 2002, Lall et al. 2006, Ďurkovič and Mišalová 2009, Dinçer et al. 2016).

Although the micropropagation of *S. aucuparia* has been studied, which has become an important technique for the mass production of many plant species, there is currently no study regarding the use of mature seeds to produce seedlings. In the study performed by Basharuddin and Smith (1993) regarding breaking seed dormancy in an *in vitro* environment, it was stated that a tissue environment which had auxins present accelerated the greening of excised embryos of *S. aucuparia* cotyledons. However, this study failed to explain the germination of excised embryos or seeds, their development into seedlings nor their growth conditions. Lall et al. (2006) evaluated different explants of *S. aucuparia* L. by first excising zygotic embryos from immature fruits and later used the somatic embryos developed in their cotyledons as their material. However, low germination rates were reported, and it was identified that further studies would be required to increase the germination rate through somatic embryogenesis. Other studies, for example, carried out by Chalupa (2002), used nodal segments and shoot tips obtained from different parts of mature *S. aucuparia* L. trees as explants (i.e. from the branches growing 2 m high, from the top branches of trees or younger parts of trees – epicormic shoots) and reported high success rates from younger plants. In another study, Emam and Ghamarizare (2013) used the nodal segments and shoot tips from mature *S. aucuparia* L. trees as explants and subjected them to 12 months of pre-treatment for rejuvenation. Studies conducted on different *Sorbus* species used similar explants. In contrast, Bekçi (2010) used direct mature seeds from *Sorbus torminalis* L. Crantz alongside shoot explants and identified the best results at 3 mg/L BAP, with any increases and decreases in BAP dosing reducing the success rate.

The aim of this study was to remove the germination barrier in an *in vitro* environment and determine the most suitable plant growth regulators for micropropagation of *S. aucuparia* L. seeds as potential production material. For this purpose, *S. aucuparia* L. seeds containing mature embryos were cultured *in vitro* in MS mediums containing different plant growth regulators.

Materials and methods

Plant materials

The *S. aucuparia* L. seeds were collected in the first half of October from healthy and mature trees growing in their natural environment in the Eastern Black Sea region of Turkey at the elevation of 1,200 to 1,300 m a.s.l. BES. The seeds were removed from the flesh of the fruits, cleaned under running tap water and left to dry at room temperature.

For the surface sterilization of the seeds, the seeds were first washed under tap water, placed in a sterile cabin (Nüve MN 120) with 70% ethanol solution for 30 seconds. Following this, the seeds were placed in solution of

3% NaOCl and 0.5% Tween-20 for 15 minutes. After this process, surface sterilization was completed by passing the seeds through sterilized distilled water 3 times for 3 minutes at a time. The sterilized seeds were then peeled with the use of sterile scalpels and forceps, and within the same week, cultured vertically (hypocotyls remaining in the medium) in glass test tubes containing different doses and combinations of plant growth regulators (3 mg/L BA + Kinetin (0.5, 1, 2 mg/L), 3 mg/L BA + IBA (0, 1, 0.5, 1 mg/L) and 3 mg/L BA + NAA (0, 1, 0.5, 1 mg/L)) in a Murashige and Skoog (MS) medium. Once every 3–4 weeks, the uncontaminated and live explants were subcultured in newly prepared mediums.

Preparation of mediums and effects of plant growth regulator (PGR) combinations

In similar studies we conducted on different plants belonging to the same family, it was observed that 3 mg/L of BA produced the best results (Dinçer 2010, Dinçer et al. 2022). As a result, experiments were carried out in a MS medium containing different doses and combinations of Kinetin (0.5, 1, and 2 mg/L), IBA (0.1, 0.5, and 1 mg/L) and NAA (0, 1, 0.5, and 1 mg/L) to break seed dormancy, whilst keeping BA constant at 3 mg/L. In addition, in all the experiments, 30 g/L sucrose was added to the medium and the pH was maintained at 5.7 to 5.8. After adding 6 g/L agar to solidify the medium, it was autoclaved at 121°C and 1.05 kg/cm^2 pressure for 15–20 minutes.

As the incubation medium for the cultivated explants, the climate cabinet was set to 23 ± 1°C and 70% humidity with white fluorescent light (80 µmol m^{-2} s^{-1}) at 16-hrs of light and 8-hrs of dark. All chemicals were purchased from Sigma Chem. Co.

Rooting and transplantation

Microshoots (≥ 1.5 cm) obtained in subculture in MS underwent a two-step rooting method. They were first kept in a MS/2 root induction medium containing different doses and combinations of IBA (0, 1, 0.5, and 1 mg/L), IAA (0, 1, 0.5, and 1 mg/L), and NAA (0, 1, 0.5, and 1 mg/L) for 2 weeks and later transferred to a MS/4 medium without any auxins. After 6 to 8 weeks, the percentage of rooted shoots was recorded. The rooted shoots were then transferred to plastic pots with a mixture of peat : sand : pearlite (2 : 1 : 1). The pots were covered with plastic film and placed in the climate cabinet containing culture conditions. All shoots transferred to soil were irrigated with $^1/_8$-strength MS inorganic salts (at an interval of 2–3 days) (Dai et al. 2007). The pots placed in the climate cabinet were gradually opened and after 3 weeks, they were transferred to a greenhouse environment with a temperature of 25°C and given 12-h photoperiod light. The soiled seedlings which were taken into the greenhouse environment were then covered with perforated covers. The covers were then gradually opened, and after 2 weeks opened completely. After being kept uncovered in a greenhouse environment for a further 2 weeks, they were taken outside.

Experimental design and data analysis

The experiments were set up in a completely randomized design, and each PGR combination was evaluated three times with a minimum of 30 to 35 explants per evaluation. The survival rate was calculated by determining the number of seeds germinated after 2 weeks among all cultivated explants in proportion to the total number of explants (number of germinated explants/total number of explants × 100). The average shoot number was determined by counting the number of shoots propagated in germinated explants after 10 weeks in proportion to all germinated explants (number of shoots in germinated explants/number of germinated explants). The average shoot length was determined by measuring the length of all shoots proliferated after 10 weeks in proportion to the number of proliferated explants (length of all proliferated shoots/number of proliferated explants). The average number of nodes was determined by counting the nodes formed in the shoots after 10 weeks in proportion to the total number of shoots (number of nodes formed on shoots/total number of shoots). The rooting percentage was calculated by counting the number of germinated seeds showing root formation after 2 weeks in the rooting medium in proportion to the total number of germinated seeds (number of seeds rooted in the germination medium/total number of germinated seeds × 100). The rooting rate was calculated by counting the number of shoots rooted in the root induction medium after 8 weeks in proportion to the total number of shoots (number of rooted shoots/total number of shoots × 100).

Statistical methods were used for evaluating the results included for assessing kinetin, IAA, and IBA hormones applied to plant samples. Several analyses were conducted to reveal the relationship between kinetin hormones and the plants upper parts, namely, shoot length, number of shoots, and number of nodes. In addition, we investigated the effects of IAA and IBA on the plants rooting.

Several statistical analyses were used as tools in this study. In statistical analyses, the first step is to assess data eligibility and normality. Hence, utilizing a proper test is essential (Hollander and Wolfe 1999, Demir 2020). As such, data eligibility was evaluated using the Chi-Square test, and a relationship was found. Mainly, there are two principal analysis methods used, parametric and non-parametric tests. The parametric test is suitable if data is normally distributed; and otherwise, a non-parametric alternative must be preferred (Coşkun et al. 2017, Demir 2017). To determine whether data is normally distributed or not, the Shapiro-Wilk normality test was conducted using SPSS Statistics, version 21.0 (IBM 2012) and found no normality. For this reason, a non-parametric variance analysis, i.e. the Kruskal-Wallis test (Kruskal and Wallis 1952), was preferred to test the hypotheses with a 95% confidence interval.

To summarize, the chi-square, Shapiro-Wilk, and Kruskal-Wallis tests were used respectively to analyse the effects of kinetin hormone (at 0.5, 1, and 2 mg/L) on the average shoot length, number of shoots, and number of nodes, and the impact of IAA and IBA hormones (at 0, 1, 0.5, and 1 mg/L) on rooting with the following hypotheses. The same hypotheses were evaluated separately for all PRGs.

Hypothesis: There is a relationship between the doses of PGR and the plants survival, shoot length, number of shoots, number of nodes, and rooting.

Results

Germination and shoot regeneration

It was determined that none of the MS mediums containing combinations of 3 mg/L BA + IBA (0, 1, 0.5, and 1 mg/L) nor 3 mg/L BA + NAA (0, 1, 0.5, and 1 mg/L) had any impact on germination and shoot regeneration, with all seeds losing their vitality at the end of 4 weeks.

In all the MS mediums containing 3 mg/L BA + kinetin (0.5, 1, and 2 mg/L), cotyledons began to open and started to germinate after 1 week, although the number of cotyledons varied in each culture medium (Figure 1a, 1b). Additionally, it was observed that hypocotyls started to grow in length in some mediums. At the end of 2 weeks, 25% of the explants in the germination medium developed shoots and directly formed roots, although the number of roots varied between 1–2 (Figure 1c, 1d). All germinated explants continued to grow healthily. In the culture initiation medium, it was observed that the rowanberry seeds (which have germination limitations) were able to begin germination within 1 week. In the explants that were placed in new media every 3–4 weeks, it was observed that shoot propagation of these explants rapidly increased at the end of 4 weeks (Figures 1e, 1f).

Effects of cytokinins on germination

After 2 weeks, all explants that germinated and grew shoots were counted and recorded to determine the germination rate. The chi-square test revealed that kinetin doses (p-value [0.000] < α [0.05]) at a statistical significance with a 95% confidence level (Cramer V value is 0.724), had relevance to the numbers of germinated explants. Increases and decreases in kinetin doses reduced the germination rate. The impact kinetin on the germination rate in the MS mediums were recorded as 96% in 3 mg/L BA + 1 mg/L kinetin doses, 43% in 3 mg/L BA + 2 mg/L kinetin doses, and 8% in 3 mg/L BA + 0.5 mg/L kinetin doses (Figure 2). As a result of this study, the MS medium with 3 mg/L BA + 1 mg/L kinetin was determined to be appropriate for the germination of rowanberry seeds in a short period of time.

Figure 1. Stages of the study: **1a** On day 3, the cotyledons opened in the seeds cultured in MS medium containing 3 mg/L BA and 1 mg/L kinetin; **1b** At the end of 1 week, germination started in the seeds cultured in MS containing 3 mg/L BA and 1 mg/L kinetin; **1c** At the end of 3 weeks, shoot proliferation occurred in the seeds cultured in MS containing 3 mg/L BA and 1 mg/L kinetin; **1d** At the end of 3 weeks, surprise rooting occurred in the shoot propagation MS medium containing 3 mg/L IBA and 1 mg/L kinetin; **1e**, **1f** After 4 weeks, shoot growth was observed in both rooted and rootless explants in the shoot propagation MS medium containing 3 mg/L BA and 1 mg/L kinetin; **1g**, **1h** After 8–10 weeks, shoot elongation and shoot proliferation was observed in the shoot growth MS medium containing 3 mg/L BA and 1 mg/L kinetin; **1i**, **1j** Root growth observed in micro shoots kept in a MS/2 medium containing 1 mg/L IAA for 2 weeks and later moved to a MS/4 medium for 4–8 weeks; **1k**, **1l** Root formation was observed in micro shoots kept in the MS/2 containing 0.1 mg/L IBA for 2 weeks and transferred to MS/4 for 4–8 weeks; **1m** Root development was observed in seedlings successfully transferred in ex vitro conditions at the end of 6 weeks.

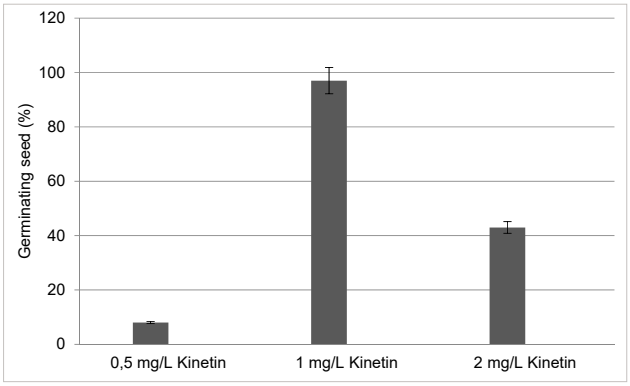

Figure 2. Germination rate of explants in the MS medium with BA + kinetin after 2 weeks

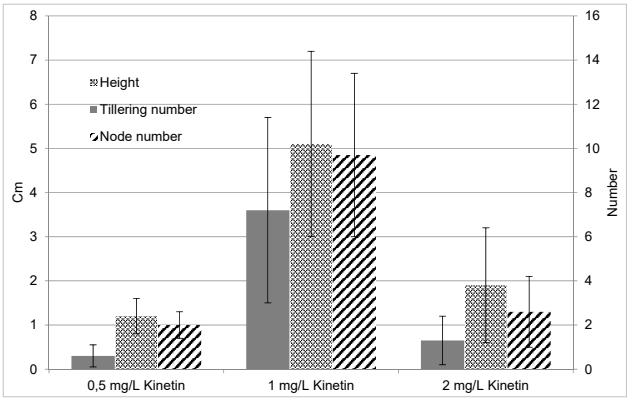

Figure 3. The impact of different doses of kinetin on cultures of *Sorbus aucuparia* L. at the end of 10 weeks in MS with respect to the average number of nodes, average number of shoots and average shoot length. The bars presented represent standard deviation

The impact of cytokinins on the number of shoots, shoot length, and number of nodes

After germination, as shoot lengthening and shoot propagation occurred in the culture initiation medium, propagated shoots were subcultivated in the same medium. The number of nodes, shoot lengths, and the number of shoots, i.e. shoot propagation were recorded after 10 weeks (Figure 1g, 1h). As the data obtained was not normally distributed according to the Kolmogorov-Smirnov and Shapiro-Wilk tests ($p < 0.05$), the Kruskal-Wallis test was performed (p [0.000] < 0.05), and a statistically significant difference was observed at a 95% confidence level regarding the effects kinetin doses have on shoot lengths, number of shoots, and the number of nodes. When the two comparisons are examined, a significant difference was observed at a 95% confidence level for kinetin doses between 0.5 mg/L and 1 mg/L and kinetin doses between 1 mg/L and 2 mg/L with respect to shoot lengths, number of shoots, and the number of nodes (Table 1).

It was observed that increases and decreases in kinetin doses reduced the success rate, and the highest values were obtained from a MS medium containing 3 mg/L BA + 1 mg/L kinetin, with an average shoot length of 5.1 cm, average number of shoots of 7.2, and average number of nodes of 9.7 (Figure 3).

Rooting and transplantation

Once the microshoots (that were formed in the subcultures) reached ≥ 1.5 cm in length, they were transferred to a MS/2 rooting medium containing auxins, which was the first step of a two-stage rooting method. After 2 weeks, as the second step of the rooting method, they were transferred to an auxin-free MS/4 medium. In the first stage of the rooting method, no rooting was observed in the media containing NAA, despite the dense callus formation. In the mediums containing IAA and IBA, rooting was recorded at different percentages (Figure 4). For microshoots formed in the subcultures, rooting time is usually 6 weeks but could extend up to 8 weeks in some instances. The most successful result (62%) was observed in MS/2 with 1 mg/L IAA (Figure 1i, 1j). It was observed that a decrease in the IAA dose decreased the rooting rate. The chi-square test (p [0.000] < α 0.05) at a 95% confidence level showed a moderate relationship between IAA doses and rooting.

The second most effective plant growth regulator in rooting microcuttings was observed to be 0.1 mg/L IBA in MS/2 with a rooting rate of 28% (Figure 1k, 1l). Unlike the IAA, decreases in the IBA doses was observed to increase the rooting rate. The chi-square test (p [0.001/2] < α [0.05]) at a 95% confidence level indicated a moderate relationship between the IBA doses and rooting of microcuttings (p [0.000] < α [0.05]), which was found to be significant. In terms of rooting speed, IBA (4 to 5 weeks) was observed to be more effective than IAA (6 to 8 weeks). The microshoots which rooted in both propagation and rooting mediums were successfully transferred to an *ex vitro* medium (Figure 1m). By ensuring that the seedlings (which were

Table 1. The impact of different doses of kinetin at the end of 10 weeks on shoots formed from the regeneration of seed explants of *Sorbus aucuparia* L. with respect to shoot length, the number of shoots and the number of nodes (the Kruskal–Wallis or chi-square multiple-range test (p [0.000] < 0.05)

Shoot length		Shoot number		Number of nodes	
Kinetin dose *	Mean ± *Std.* Deviation **	Kinetin dose *	Mean ± *Std.* Deviation **	Kinetin dose *	Mean ± *Std.* Deviation **
0.5 mg/L	1.22 ± 0.6 [a]	0.5 mg/L	0.63 ± 0.5 [a]	0.5 mg/L	2.00 ± 0.9 [a]
1 mg/L	5.27 ± 2.1 [b]	1 mg/L	7,42 ± 4.2 [b]	1 mg/L	9.98 ± 3.6 [b]
2 mg/L	1.89 ± 1.3 [a]	2 mg/L	1.33 ± 1.3 [a]	2 mg/L	2.56 ± 1.4 [a]

Notes: * 100 samples were evaluated for each dose. ** Differences between the values are shown as separate groups using superior characters *a* and *b*.

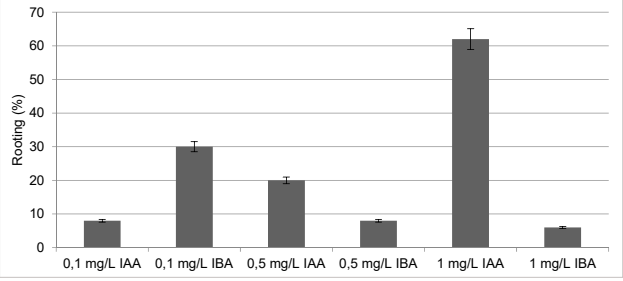

Figure 4. Rooting rate of microshoots that were kept in a MS/2 rooting medium containing the different doses of IAA and IBA for 2 weeks and transferred to the auxin-free MS/4 medium for 6–8 weeks

in pots of soil) were covered and opened gradually during their time in the climate cabinet and in the greenhouse, all of the plants survived. There was no loss at this stage.

Discussion

Like all forest trees, seed production is very important for the propagation of the *Sorbus* species and the breeding of novel cultivars. Separately, the sexual reproduction of the *Sorbus* species protects genetic diversity, whereas clonal propagation protects its genetic diversity for an extended period (Hoebee 2006). *Sorbus aucuparia* L., which has several horticultural, agricultural, forestry, medicinal and industrial applications as they are generally produced by seeds. However, *Sorbus* species have germination-related problems due to embryo dormancy, with seeds requiring 9-to-26-week of cold stratification to germinate (Devillez 1979, Devillez et al. 1980, Lenartowicz 1988, Chalupa 2002, Paulsen et al. 2002, Paganova 2007, Var 2010, Drvodelic 2018). Compared to previous studies, this study was able to break the dormancy of wild *S. aucuparia* L. seeds in the shortest time, with germination occurring in 1 week, and shoot growth occurring in 2 weeks. Similarly, the observed germination rate of 96% (which occurred in 1 week) was also the highest rate when compared to germination rates reported in the previous studies.

Unlike stratification, Nikolaeva et al. (1987) reported that kinetin (10 mg/L), gibberellic acid (100 mg/L) and thiourea (7.5 g/L) may be effective in breaking seed dormancy of *S. aucuparia* and stimulating germination of isolated embryos, but was ineffective when the perisperm was intact, even if the test was removed. In our study, it was observed that kinetin in combination with BA (rather than alone) was effective in breaking the seed dormancy of *S. aucuparia*. Separately, removing the seed coat of the *S. aucuparia* seeds reduced the cold stratification time required for germination. However, the removed embryos still required a short period of treatment (Devillez et al. 1980).

Devillez et al. (1980) reported that the removal of the seed coat of *S. aucuparia* did not break dormancy but reduced the cold stratification time required for seeds to germinate. In our study, the removal of the seed coat was effective in shortening the germination period. Following successfully breaking seed dormancy, rowanberry seeds may germinate precociously (or prematurely) during pre-treatment, which may lead to deterioration and germinated seeds are prone to damage during sowing operations (Tanaka 1984). In the method that we developed, this problem is completely eliminated as the seedlings are rooted one by one and sown into soil. The results obtained in this study may be beneficial for the mass production of forest trees via micropropagation (whose seed production was limited due to dormancy), the breeding of novel cultivars, the protection of forest species and for reforestation.

The vegetative propagation capacity of trees decreases with age. Thus, mature trees usually have a low vegetative propagation capacity. Micropropagation, in contrast, yields the most promising results from young plants (McCown 2000, Chalupa 2002, Nas and Read 2004b). As a result, techniques such as heavy pruning, grafting mature shoots onto young seedling rootstocks, spraying plant with growth regulators to stock plants, and using a forcing solution can regenerate mature trees to increase the response of explants for micropropagation (Meier and Reuther 1994, Vieitez et al. 1994, Chalupa 2000, Preece and Read 2003). Lall et al. (2006) used shoot tips taken from mature *S. aucuparia* L. trees as explants and reported that the number of shoots only increased after the fifth subculture at the first stage of their micro propagation study due to the plant maturity. However, in our study, very successful results were obtained for the first subculture and highly effective shoot elongation was achieved by using the seeds collected from mature plants as a direct material without any pre-treatment. This can be an important advantage in shortening the production time for both seed production and micro propagation. During the second stage of their study, Lall et al. (2006) subjected zygotic embryos isolated from immature fruits to pre-treatment at 4°C and then used them as explants for somatic embryo formations. This occurred in approximately 10 months and a 20% germination success rate was reported. Krajňáková et al. (2008) also reported that embryo transformation and germination were the most difficult stages for several plant species. In the present study, a 96% germination rate was achieved within 1 week, and shoot growth was observed after 2 weeks using mature seeds that were not subject to any pre-treatment.

Plant growth regulators have an impact on shoot propagation and shoot lengthening (Ruzic and Vujovic 2008, Nas et al. 2012). Among cytokinins, the most frequently used plant growth regulators in micro-propagation that stimulate cell division and shoot growth are BAP and kinetin (Phillips and Garda 2019). Previous studies on micropropagation of the *Sorbus* L. species indicate that a combination of BAP or TDZ with an auxin is used as a cytokinin (Chalupa 2002, Mala et al. 2009, Yang et al. 2012, Emam and Ghamarizare 2013). Only one study used kinetin, more specifically a combination of kinetin and BAP as

well as an auxin (Lall et al. 2006). To our knowledge, no studies used a combination of only cytokinins. In the present study, a combination of only two cytokinins (BAP and kinetin) without any auxins was used during germination and a high-rate success was observed with respect to shoot growth. In this study, compared to previous studies, the highest number of shoots and the highest germination levels were obtained in a considerably shorter amount of time.

It has been observed that IBA is generally used as an auxin in the rooting of microshoots of the *Sorbus* species (Chalupa 2002, Mala et al. 2005, Yang et al. 2012, Emam and Ghamarizare 2013). In the two-stage rooting method of our study, rooting of 28% was observed in IBA, while a higher rate of 62% was achieved in IAA. In addition, rooted shoots (25%) formed in the initial propagation medium can be an advantage in the production of *Sorbus* species as they have developed in the shortest time.

Conclusion

This tissue culture protocol assists with the removal of the germination barrier in a short period of time, aiding seed production, which is highly important for the propagation of the *Sorbus* species and for the breeding of novel cultivars (in a similar vein for all forest trees). In addition, it assists with clonal propagation by creating an effective and productive environment for seeds, with a reasonable amount of shoot production and rooting rate. The results presented in this study can be used for the clonal production of selected mature *Sorbus* species grown in natural environments through quicker germination periods, creating potential value for applications in forestry, pharmaceutical, industrial, ornamental, food and ecological conservation areas. It is recommended that an MS medium containing 3 mg/L BA + 1 mg/L kinetin is used to break the seed dormancy of *Sorbus aucuparia* L. *in vitro* as well as to ensure shoot elongation and proliferation. For rooting specifically, a two-step method is recommended with a MS/2 medium containing 1 mg/L IAA followed by a MS/4 medium without plant growth regulators. As such, the method revealed in this study may be important for the effective clonal propagation of seeds, for not only the *Sorbus* species, but also for similar tree species. As such, this study may have many uses in our world where the ecological balance is gradually deteriorating.

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