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ORIGINAL ARTICLE

The impact of sucrose and 6-benzylaminopurine on shoot propagation and vitrification in *Aronia melanocarpa* (black chokeberry)

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Abstract

Vitrification is one of the most significant issues encountered in plant tissue culture applications. It diminishes the quality of in vitro plants, causing their leaves and stems to appear watery and translucent, and it may impede the success of the acclimatization step. In this respect, this study investigates the impact of sucrose and 6-Benzylaminopurine (BAP) concentrations on shoot regeneration and vitrification development in *Aronia melanocarpa*, known for its high antioxidant content and health benefits. Initially, the presence of BAP, in combination with varying sucrose concentrations, leads to a substantial increase in shoot number, and the largest number (7 shoots per nodal explant) was observed in the Murashige and Skoog (MS) medium containing 3% sucrose and 5.0 mg/L BAP. Furthermore, sucrose concentration plays a crucial role in shoot growth, with higher concentrations promoting more extensive shoot development. However, when 3% sucrose was combined with higher BAP (from 1.0- to 5.0 mg/L), an increased incidence of vitrification was observed over time. Interestingly, lower sucrose concentrations (1% or 2%) combined with 0.5 mg/L or 2.5 mg/L BAP initially delayed vitrification but eventually led to its occurrence. Microscopic analysis of leaf samples with varying levels of vitrification indicates significant differences in the density of stomata, further confirming the detrimental impact of vitrification on cellular structures and physiological processes. The recovery of vitrified plants was evaluated using different growth media combinations. The absence of BAP in the medium led to higher recovery percentages (min 96%) without necrosis, while the addition of 0.5 mg/L BAP promoted shoot growth but potentially inhibited root development. It has been found that media with 1 mg/L BAP and either 10 g/L or 20 g/L sucrose, as well as media with 30 g/L sucrose and 0.5 mg/L BAP, are the most suitable for efficient shoot regeneration with minimal vitrification risk. However, increasing BAP levels for faster shoot regeneration also raises the risk of vitrification. During acclimatization, vitrified plants exhibited stunted shoot growth, shorter and narrower leaves, reduced root numbers and lengths, and decreased survival rates, particularly under lower humidity conditions. The cultivation period required for the recovery of the crop was determined to be 6 weeks under greenhouse conditions for a sustainable plant propagation.

Key message

Optimizing *Aronia melanocarpa* shoot regeneration: Balance sucrose and BAP for vigorous growth. Beware vitrification! Tailor medium and conditions for successful recovery.

Keywords *Aronia melanocarpa* · Black chokeberry · Vitrification · Shoot regeneration · 6-benzylaminopurine (BAP) · Sucrose

Introduction

Aronia, a plant species within the genus *Aronia* of the Rosaceae family, which includes here are three species: black chokeberry (*A. melanocarpa* (Michx) Elliot), purple chokeberry (*A. prunifolia* (Marsh), and red chokeberry (*A. arbutifolia* (L.) Elliot) (Šnebergrová et al. 2014). Notably, *Aronia melanocarpa* can grow up to 1.2−2.4 m high. Aronia berries have been identified as exhibiting multiple

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bioactivities that could be advantageous to human health, with key compounds including cyanidin-3-O-galactoside, chlorogenic acid, quercetin, and ursolic acid (Ren et al. 2022).

This study presents a comprehensive literature review on the micropropagation of *A. arbutifolia* (red chokeberry) and *A. melanocarpa* (Michx.) Elliot (black chokeberry). Brand and Cullina (1992) reported that shoots were placed in MS and WPM media containing 3% sucrose, 0.65% agar, and different concentrations of BA (up to 2.5 mg/L) for shoot multiplication for 8 weeks. Successful shoot multiplication and rooting of both *Aronia* varieties were achieved, and their acclimatization was reported. Similarly, Szopa and Ekiert (2014) reported that the micropropagation of *A. melanocarpa* was carried out in MS medium with seven different concentrations of BAP and NAA to establish shoot and callus cultures. Çelebi et al. (2018) reported a study to develop clonal propagation protocol for three *Aronia* varieties (Eastland, Viking, Nero) using two multiplication media. Rusea et al. (2018) investigated the effects of different concentrations of BAP (up to 10 mg/L), 2,4-D (up to 1 mg/L), and IBA (up to 1 mg/L) in various combinations on shoot regeneration of Nero, a black chokeberry variety of *Aronia*. Noteworthy, no evidence of vitrification, a physiological disorder that affects tissue culture plants, has been reported in these studies. In this respect, Tigrel et al. (2022) reported that the micropropagation of this crop at a commercial scale, involving successive subcultivations every 2 months for 4 years, was associated with vitrification. Regrettably, attempts to acclimatize approximately 10,000 vitrified clones of *Aronia* in the greenhouse were unsuccessful, attributed to inadequate care.

Vitrification is one of the most important problems encountered in plant tissue culture applications. Vitrified plants are characterized by their leaves and stems having a watery and translucent appearance. The leaves of vitrified plants are usually dark green, thick, curled, and elongated. The stems of these shoots are also thick and brittle (Rodríguez et al. 2012). It is sometimes difficult to continue propagation and rooting of vitrified plants and transfer them from culture vessels to soil (Bonga et al. 1992). Additionally, they are more susceptible to infections and wilt quickly (Joyce et al. 2003). Therefore, this problem can lead to yield loss, especially in commercial production. Vitrification has been reported to cause abnormal development of leaf, stem, and root physiology, along with weak palisade parenchyma and cuticle layer in the basic tissues of plants. The mechanisms underlying vitrification, also referred to as *hyperhydricity*, remain incompletely understood. It has been reported that increasing agar, gelrite, and sucrose concentrations, altering macroelement mixtures, and reducing relative humidity by ventilating the culture vessel decrease the incidence of vitrification in plants (Polivanova and Bedarev 2022). Ziv et al. (1983) transferred plants to solid media after growing them in liquid culture for several days to reduce the vitrification in carnation plants. They stated that preference of solid media after liquid was effective in reducing vitrification. Huang et al. (1995) observed vitrification in both gelrite and agar media, and they reported that increasing gel concentration minimized vitrification. Some studies also indicate that relative humidity in culture vessels plays a role in the occurrence of vitrification (Zimmerman et al. 1991; Kataeva et al. 1991; Pierik 1997; Gribble 1999; Sangwan and Sangwan-Norreel 2012). It was reported that increasing agar from 6- to 12 g/L reduced vitrification but also decreased plant growth (Hakkaart and Versluijs, 1983). Pasqualetto et al. (1988) studied the effect of potassium, magnesium, and gelling agent concentrations on vitrification, a study was conducted on shoot tips of 'York' and 'Vermont Spur Delicious' apple trees (*Malus domestica* Borkh.) cultured in vitro. In *Gypsophila paniculata*, Dillen and Buysens (1989) found that omitting $FeSO_4$ and Na_2EDTA from the medium and using a high concentration of agar (9 g/L) resulted in a high incidence of vitrification in the plants. Alqadasi et al. (2022) reported the excessive cytokinin concentrations (i.e. 3.0 mg/L or more BAP) may lead to issues like vitrification and genetic instability in micropropagated ginger plants.

This complex phenomenon influenced by various factors needs to be optimized to improve the success of plant tissue culture techniques, and the present study aimed to investigate the effects of sucrose and BAP, the most frequently used cytokinin in tissue culture systems on the regeneration and vitrification of *A. melanocarpa* explants. In the literature, limited research has been conducted on the effects of different concentrations of BAP and its interaction with sucrose in relation to vitrification in tissue culture systems, as well as its impact on the rooting and hardening processes. The findings of this study offer valuable insights into the factors influencing the growth and development of *A. melanocarpa* as a model crop and draw attention to the vitrification during large scale production systems in vitro.

Materials and methods

Selection of plant material

The explants of Nero variety of *Aronia melanocarpa* were harvested from trimmed branches with sprouting fresh shoots excised from 2 year old mature bushy trees in the field. Meristematic nodal explants were cut into 2.5 cm long pieces and were surface sterilized following the protocol described in Tigrel et al. (2022). Briefly, the fresh shoot explants, after sprouting from the nodes on each branch, were dipped into 70% ethanol (v/v) for 1 min, followed by dipping in a 15% (v/v) of commercial bleach solution

(Domestos® containing 5% sodium hypochlorite) for 20 min. After rinsing the explants with autoclaved water, the nodal explants were transferred to the growth medium.

Preparation of shoot regeneration and vitrification induction media

MS medium with vitamins (4.4 g/L; Duchefa, The Netherland) in combination with four different sucrose concentrations (0-, 10-, 20-, or 30 g/L) and five different BAP concentrations (0-, 0.5-, 1.0-, 2.5-, or 5.0 mg/L) were tested (see from M1 to M15, in Fig. 1). The medium was solidified using 3.5 gr/L Gelrite (Duchefa, The Netherland) at pH 5.7 prior to autoclaving at 121 °C. Experiments were conducted in glass jars (330 ml) with semi-transparent plastic lids. Each experiment included nine explants, with three replicates and three explants per glass jar (a total of 180 explants). Cultures were kept in a growth chamber (20 m^2) with a 16 h photoperiod from cool white-fluorescent lamps (Master TL5 HE 14 W T5, Philips, The Netherlands), with an irradiance of ~50 µmol/m^2/s (3000 ± 150 l×) frequently checked by a light meter (Testo 545, Germany) on each shelf, at 25- and 35% humidity and 24 °C. In vitro cultivation was performed for 65 days, during which various growth parameters such mean number of shoots, shoot length and callus size were recorded.

The vitrification pattern was assessed by examining the leaf morphology of at least one individual shoot. It was categorized as low, high, or none, regardless of the growth media used. Thus, high vitrification was characterized by leaves that were completely inwardly curled, exhibiting a watery/glassy appearance, dark green color, and firm texture (see details in Fig. 3a, b and c). On the contrary, leaves that exhibited a mild inward curl or no curl at all showed minimal signs of vitrification, presenting a translucent and wet appearance with a light green color (see Fig. 3d, e, and f).To induce and confirm it with microscopy, an additional 65 days of cultivation were conducted using selected growth media supplemented with various sucrose (1-, 2- or 3%) and BAP (0.5-, 1.0- and 5.0 mg/L) combinations in a total of seven treatments (Table 1). Vitrification rates were recorded on the 20th, 23rd, 30th, 38th, 44th, and 65th days of cultivation. To describe the pattern, lower and upper epidermal tissues of randomly selected leaves were examined using a light microscope (Leica ICC50 HD, Germany) at a magnification of 20×. Stomata observation (mean number of stomata cells per mm^2, width and length in Table 2) was performed to analyze the occurrence of vitrification using a computer program (Leica, LAS EZ v3.4). Following the vitrification period (max 65 days), the vitrified shoots (three shoots in each of the three replicates) were transferred to recovery media, that consisted of different sucrose concentrations (0, 1-, 2-, or 3%) with or without 0.5 mg/L BAP in a total of eight different combinations (Table 3). The shoots were placed in jars kept as above. The subsequent growth and development of shoots were observed over a period of 31 days.

Acclimatization

The roots of randomly selected healthy *Aronia* shoots were carefully washed to remove any growth medium residues. Subsequently, the shoots were placed in pre-soaked fertilizer-free peat and perlite mixture (1:10) of paper pots (70 mm × 90 mm; Agripot, Türkiye). Each paper pot was placed inside a transparent plastic culture vessels (cubic vessel 400 ml, Xplant Co., Türkiye), and 5 ml of water was added to each box. The transparent lids of the culture

Table 1 Vitrification ratios (ranging 0–77%) for each combination of media on the corresponding day

Media*	20th day	23rd day	30th day	38th day	44th day	65th day	Overall vitrification*
1% Sucrose + 0,5 BAP	0%	0%	11%	11%	11%	22%	Low
2% Sucrose + 0,5 BAP	0%	0%	22%	22%	22%	22%	Low
2% Sucrose + 1 BAP	0%	22%	22%	22%	22%	22%	Low
2% Sucrose + 5 BAP	0%	22%	22%	33%	55%	66%	High
3% Sucrose + 0,5 BAP	0%	0%	0%	0%	0%	11%	Low
3% Sucrose + 1 BAP	0%	11%	33%	44%	55%	55%	High
3% Sucrose + 5 BAP	0%	11%	22%	22%	44%	77%	High

Benzyl-amino-purine (BAP) concentrations are mg/L. Overall vitrification status was commented upon after 65 days of cultivation

Colors of the cells also sign the degree of vitrification

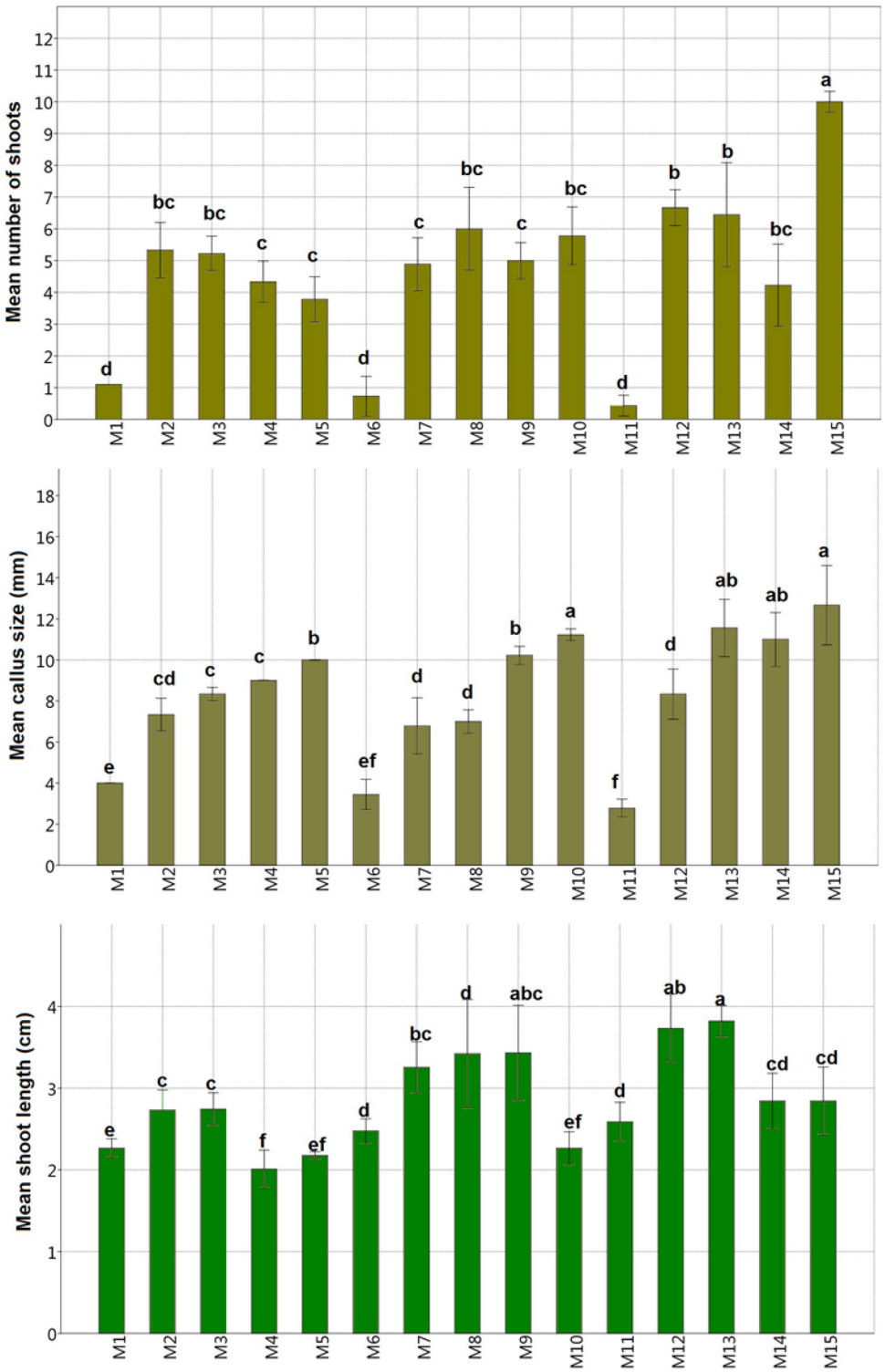

Fig. 1 Effects of growth media on different regeneration parameters (mean number of shoots, shoot length and callus size) after 65 days of cultivation. M1: 1% Sucrose, M2: 1% sucrose + 0.5 mg/L BAP, M3: 1% sucrose + 1.0 mg/L BAP, M4: 1% sucrose + 2.5 mg/L BAP, M5: 1% sucrose + 5.0 mg/L BAP, M6: 2% sucrose, M7: 2% sucrose + 0.5 mg/L BAP, M8: 2% sucrose + 1.0 mg/L BAP, M9: 2% sucrose + 2.5 mg/L BAP, M10: 2% sucrose + 5.0 mg/L BAP, M11: 3% sucrose, M12: 3% sucrose + 0.5 mg/L BAP, M13: 3% sucrose + 1.0 mg/L BAP, M14: 3% sucrose + 2.5 mg/L BAP, M15: 3% sucrose + 5.0 mg/L BAP. Bar lines show the confidence intervals of standard error of the mean values at %95

vessels were fully closed for 10 days. After 10 days, the lids were partially opened, leaving space at the corners of the boxes to lower the humidity inside (from 99% down to 65%) the boxes for further hardening. This condition was maintained for an additional 10 days (in total 20 days for whole acclimatization). All plants were then transferred to pots in a growth chamber with a relatively low humidity of 15–25%.

Statistical analysis

The data was analyzed using PAST 4.03 (Hammer et al. 2001) Statistical Software. Correlation tests and principal component analysis (PCA) were conducted to examine the relationships between variables. Additionally, one-way ANOVA was used to investigate the effects of the growth media on the measured parameters. Furthermore, two-way ANOVA was applied to assess the interaction between sugar and BAP concentrations separately. Mean values were further analyzed using Tukey's pairwise ad-hoc test for post-hoc comparisons.

Results

Effects of BAP and sucrose concentration on regeneration

Two-way ANOVA results reveal that both sucrose and BAP concentration have significant effects on the mean shoot number per explant (*F-value of 11.05; and P < 0.001*). This finding is also highly correlated with callus size. Figure 1 shows that MS medium without BAP but varying concentrations of sucrose resulted in the lowest number of shoots (see M1, M6 or M11in Fig. 1). A dramatic increase of mean shoot number was observed ranging between 0.5- and 2.5 mg/L BAP (min 4, max 7 shoots per explant, see M2, M3, M4, M7, M8, M9 and M10) depending on the sucrose concentrations. The highest shoot number was observed on MS medium in combination with 3% sucrose and 5.0 mg/L BAP (M15). With BAP at 5.0 mg/L, a gradual increase of sucrose concentration (from 1 to 3%) increased the shoot number significantly. The correlation coefficient between callus size and shoot number was calculated as 0.65 (Fig. 2), which indicates a moderately strong relationship between these variables. For instance, callus induction was pursued in presence of BAP, and callus formation was promoted (from 6- to 11 mm) with increasing concentrations of sucrose. Maximum callus size was obtained on 3% sucrose plus 5.0 mg/L BAP (12 mm in Fig. 1). Both shoot number and callus formation showed a relatively weak correlation with shoot length parameters during clonal propagation of *Aronia* with a correlation coefficient of 0.20. In this respect, sucrose concentration depending on BAP concentration played a critical role for the shoot growth. The highest shoot length (4 cm for M9, M12 and M13 in Fig. 1) was observed on 3% sucrose and decreased significantly with increasing BAP concentrations (from 1.0- to 5.0 mg/L). Vitrification had a clear pattern in the presence of BAP.

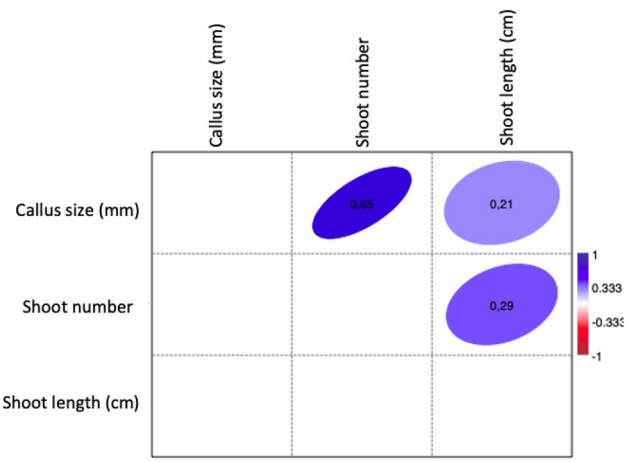

Fig. 2 Correlation analysis of different growth parameters (callus size, shoot number and shoot length). Blue color density indicates positive correlation approaching 1.0

Observation of vitrification on selected BAP and sucrose concentrations

Table 1 presents the effects of seven different regeneration media on the vitrification rate of explants at various times. On the 20th day no signs of vitrification were observed but, as the observation time progressed, results varied depending on the BAP and sucrose concentrations of the regeneration medium. Lower BAP concentrations (0.5 mg/L) combined with moderate sucrose levels (1–2%) appear to be particularly effective in low-level vitrification (max 22%) as the culture progresses. During the initial stages of culture (20th and 23rd days), MS media containing 0.5 mg/L BAP and 1–2% sucrose showed promising results, with minimal vitrification observed (*low vitrification* in Table 2). As the culture period advances (30th and 38th days), these media conditions exhibited only a slight increase in vitrification rates. Increasing BAP concentration to 1.0 mg/L led to a slight increase in vitrification rates when sucrose was fixed at 2% (22% vitrification on 23th day). The highest BAP concentration (5.0 mg/L) significantly increased vitrification rates (77% on 65th day). The most effective combination was 3% sucrose and 0.5 mg/L BAP (M12 in Fig. 1) without any sign of vitrification until 65th day. As for the assessment of vitrification status, three media were observed for the *high vitrification* (2% sucrose + 5.0 mg/L BAP, 3% sucrose + 1.0- or 5.0 mg/L BAP in Table 1), while the remaining media were considered as inducing *low vitrification.*

Identification of vitrified samples on the leaves

The data presented in Table 2 show the stomata characteristics in leaf samples with varying levels of vitrification. The leaf morphology and the mean number, width, and length of

Table 2 Stoma characteristics in leaf samples with varying levels of vitrification

Leaf morphology	Mean number of stoma cells/ mm^2 leaf area	Stoma width (µm)	Stoma length (µm)
No vitrification	76.12 ± 3.95^{c}	27.92 ± 0.39^{c}	42.21 ± 0.62^{b}
Low vitrification	106.27 ± 5.65^{b}	32.47 ± 0.60^{b}	42.73 ± 0.67^{b}
High vitrification	135.43 ± 5.32^{a}	35.52 ± 0.59^{a}	45.13 ± 0.69^{a}

Mean separation within treatments indicated by different letters, by Tukey's post-hoc test at $P \leq 0.05$

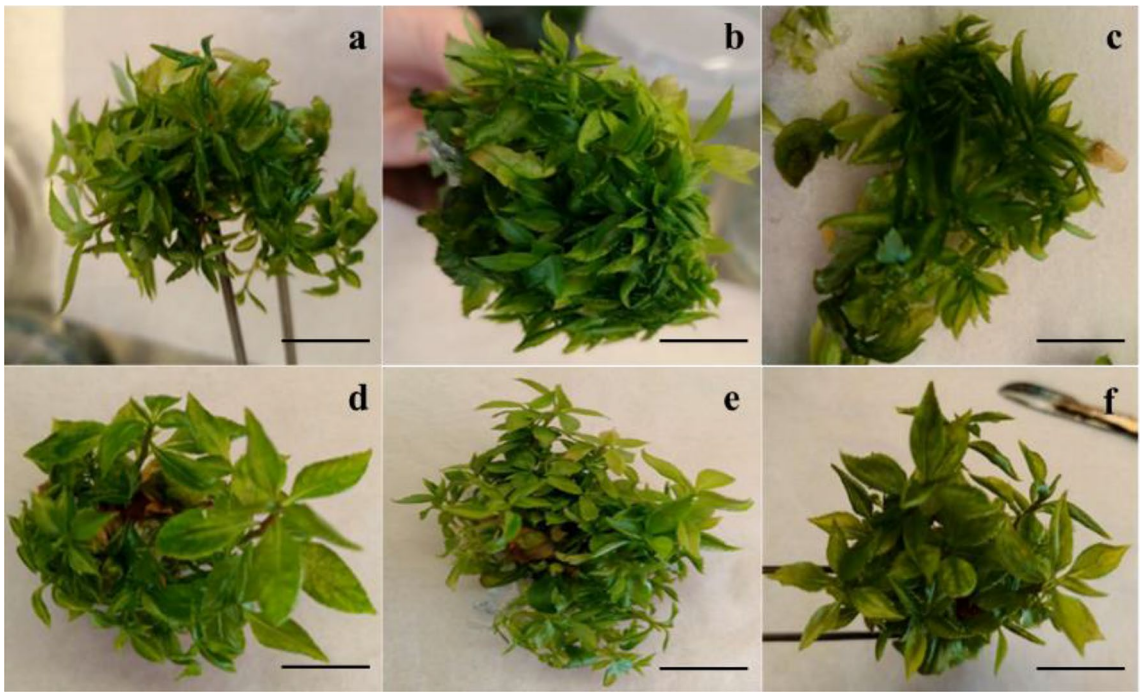

Fig. 3 Vitrification pattern in the leaves of Aronia melanocarpa. Low vitrification (**a**–**c**), and high vitrification (**d**–**f**)

Table 3 Effects of sucrose concentration and 6-benzylaminopurine (BAP) on growth parameters (mean number of shoots and roots, callus size) after 31 days of culture initiation and recovery at corresponding days (13th and 31st day of cultivation)

Growth media	Mean number of shoots	Mean callus size (mm)	Mean shoot size (cm)	Mean number of roots	% of recovery at day 13th	% of recovery at day 31st
0 Sucrose + 0 BAP	0	0	0	0	Necrosis	Necrosis
0 Sucrose + 0,5 BAP	0	0	0	0	Necrosis	Necrosis
1% Sucrose + 0 BAP	1.8 ± 0.1^{d}	6.0 ± 0.0^{c}	3.8 ± 0.4^{c}	5.7 ± 1.2^{b}	96	98
2% Sucrose + 0 BAP	1.3 ± 0.2^{e}	5.0 ± 0.1^{d}	2.8 ± 0.3^{d}	4.8 ± 0.8^{b}	96	98
3% Sucrose + 0 BAP	1.5 ± 0.2^{de}	5.0 ± 0.1^{d}	4.2 ± 0.6^{bc}	10.2 ± 0.7^{a}	100	100
1% Sucrose + 0,5 BAP	5.0 ± 0.2^{a}	10.0 ± 0.5^{a}	4.1 ± 0.5^{bc}	0.0 ± 0.0	26.1	94.3
2% Sucrose + 0,5 BAP	4.1 ± 0.3^{b}	7.6 ± 0.2^{b}	4.6 ± 0.5^{b}	2.0 ± 0.7^{d}	48.4	85.8
3% Sucrose + 0,5 BAP	3.1 ± 0.1^{c}	7.9 ± 0.1^{b}	5.5 ± 0.6^{a}	3.3 ± 1.2^{c}	66.3	83.9

Mean separation within treatments indicated by different letters, by Tukey's post-hoc test at $P \leq 0.05$. Sucrose concentration is % (w/v), BAPis mg/L. Zero values shows no shoot, root and callus formation due to the necrosis

stomata were analyzed for three different levels of vitrification: no vitrification, low vitrification (Fig. 3a, b and c), and high vitrification (Fig. 3d, e and f). This identification was based on earlier morphological observation by naked eyes, and further detailed in microscopic observations accordingly (Table 1). The microscopy results indicate that as the level of vitrification increases, there is a significant difference in the mean number of stomata cells, stomata width, and stomata length (see Table 2). In leaf samples produced from MS medium containing 3% sucrose and 0.5 mg/L BAP, the mean number of stomata cells per mm^2 was 76.12, stomata width was 27.92 µm, and stomata length was 42.21 µm indicating no vitrification. Comparatively, for the low vitrification, the mean number of stomata cells increased significantly to 106.27, their width increased to 32.47 µm, and the length was 42.73 µm (see Fig. 3a–c). Furthermore, in leaf samples with high vitrification (see Fig. 3d, e), mostly produced from 3% sucrose and 1.0- or 5.0 mg/L BAP on 65th observation day, the mean number of stomata significantly increased to 135.43 per mm^2 with a mean stomatal width of 35.52 µm, and a length of 45.13 µm (Table 2).

Effects of sucrose and BAP on the recovery of the vitrified plants

Table 3 represents the recovery percentages of vitrified plants (mostly having a sign of high vitrification on leaves) for different growth media combinations at Day 13 and Day 31, as well as the occurrence of necrosis. At Day 13, necrosis was predominant pattern for the first two growth media combinations (0 Sucrose plus 0- or 0.5 mg/L BAP, Table 3). However, for the growth media without BAP, the recovery percentages were relatively high (96% or 100%) and without any sigh of necrosis, vitrification, or tissue damage. By Day 31, no necrosis was observed, the recovery percentages of vitrified plants ranged from 83.9 to 100%. MS with 1–3% sucrose concentrations only affected significantly growth parameters. Thus, 3% sucrose shows the highest mean number of shoots (1.5), mean callus size (5.0 mm), and mean shoot size (4.2 cm). Additionally, in the absence of BAP, growth media showed the highest mean number of roots (10.2), indicating a significant impact of sucrose concentration on growth parameters. This was also significantly correlated with the recovery of vitrified plants into healthy ones with a well-developed root system. The growth media combinations with 0.5 mg/L BAP showed different results in terms of shoot number per explant and callus size but not of root number. Higher sucrose concentrations and the absence of BAP appeared to promote greater root and shoot development, while the addition of 0.5 mg/L BAP enhanced shoot growth. The highest mean number of shoots (5.0) and mean callus size (10.0 mm) occurred with 1% sucrose plus 0.5 mg/L BAP, while 2% sucrose and 0.5 mg/L BAP combination exhibits intermediate values for all growth parameters, and the combination of 3% sucrose and 0.5 mg/L BAP gave the highest mean shoot size (5.5 cm) but was engaged with the lowest percentage of recovery at 31st day (Fig. 4a).

Effects of vitrification on physiological parameters during acclimatization

In this experiment vitrified plants were transplanted onto the pots after 65 days of incubation (Fig. 4b, c; Table 4). There was no significant difference in the mean number of leaves between recovered plants (10.0) and vitrified plants (9.6) indicating that vitrification did not have a substantial impact on this trait. Vitrified plants exhibited significantly shorter (1.0 cm) and narrower (0.7 cm) leaves compared to recovered plants (leaf length: 2.4 cm, width: 1.2 cm, Table 4), and lower mean number of roots (10.4) and shorter root length (1.3 cm) than recovered plants (19.8 roots per shoot with a root length of 4.4 cm; see Fig. 4d). In terms of mean shoot length, vitrified plants exhibited a significantly shorter mean shoots (2.5 cm) compared to healthy plants (5.9 cm) indicating that vitrification severely impaired shoot growth and leads to stunted shoot development (Fig. 4d). To assess the differences between the vitrified and recovered (healthy) groups, the Principal Component Analysis (PCA) scores of PC1 (72.6%), PC2 (14.7%), and PC3 (5.9%) in Fig. 5 were analyzed. PC1 emerged as the most influential component, effectively separating the two groups, followed by PC2, which accounted for the highest amount of variance. The vitrified group exhibited predominantly negative scores on PC1, indicating a distinct pattern or characteristic differentiating them from the recovered plants. Additionally, the survival rate of the healthy plants remained at 100% under both 95% humidity and 25–35% humidity conditions. In contrast, the vitrified plants displayed a reduced survival rate of 81.8% (see Fig. 4e) at 95% humidity and nil (0%) at 25–35% humidity (see Fig. 4f). These findings highlight the vulnerability of vitrified plants to environmental conditions and their diminished ability to withstand lower humidity levels after a 20-day acclimatization period (Fig. 4g).

Discussion

Tissue culture offers the possibility of large-scale propagation, yet it comes with two major challenges: contamination and vitrification. Contamination is a problem that can be easily recognized and addressed promptly; however, its impact can be devastating if allowed to disseminate within the cultures. Therefore, it is crucial for researchers working with tissue culture systems to take all necessary precautions before initiating in vitro cloning to prevent contamination.

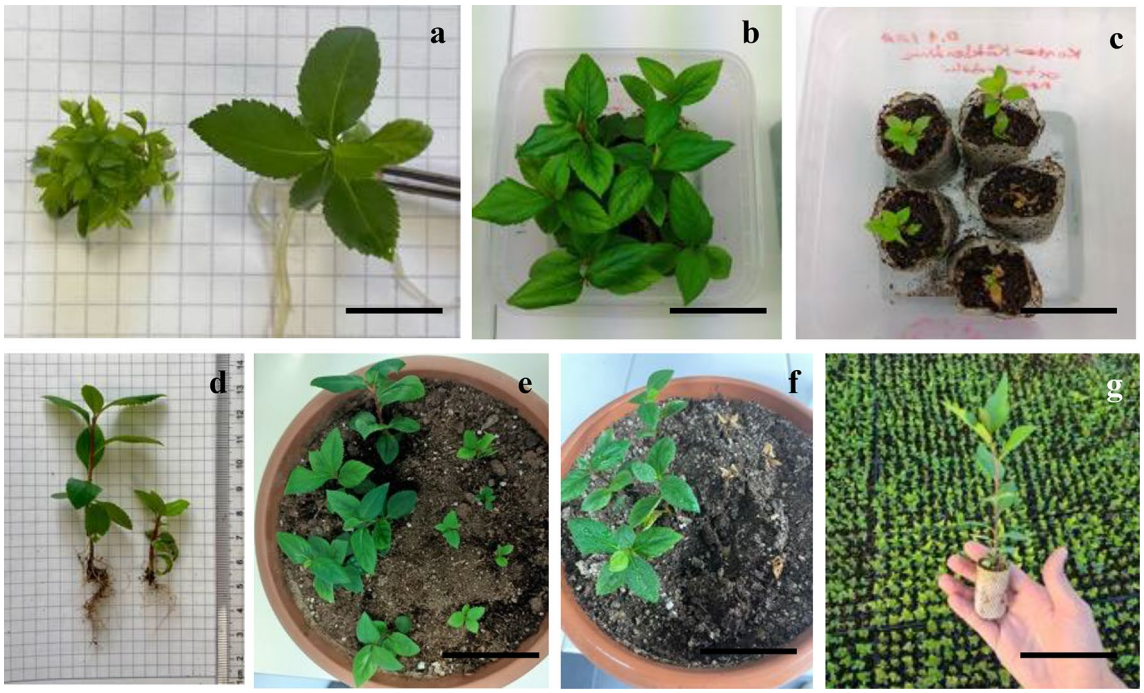

Fig. 4 Effect of 1% or 2% of sucrose in combination with 0.5 mg/L BAP on recovery rate after 13 days (left) and single shoot growth and development without clustering at basal point on 3% sucrose only (right) (**a**), recovered in vitro plants (**b**) and vitrified plants in paper pots at humidity of 95% for 10 days (**c**), comparison of recovered (left) and vitrified plants (right) after 10 days of potting at 95% humidity (**d**), transferring of vitrified- and recovered plants into large pots at relatively low humidity (min 25%, max 35%) (**e**), no vitrified plant survived (right) after 20 days of acclimatization (**f**), successful survival performance of recovered Aronia clones under greenhouse conditions after 45 days of acclimatization (**g**)

Table 4 Comparative analysis of growth parameters in healthy (or recovered) and vitrified *Aronia melanocarpa clones* following 20 days of acclimatization in paper pots

Growth parameters	Healthy plants	Vitrified plants
Mean number of leaves	$10.0 \pm 0.6^{n.s.}$	9.6 ± 0.7
Mean Leaf length (cm)	2.4 ± 0.3*	1.0 ± 0.1
Mean Leaf width (cm)	1.2 ± 0.1*	0.7 ± 0.0
Mean number of roots	19.8 ± 2.1*	10.4 ± 1.3
Mean Root length (cm)	4.4 ± 0.5**	1.3 ± 0.3
Mean Shoot length (cm)	5.9 ± 0.2***	2.5 ± 0.2
Survival rate at 95% humidity	100%	81.8%
Survival rate at 25–35% humidity	100%	0%

Significant difference at $*P \leq 0.01$, $**P \leq 0.001$, $***P \leq 0.0001$; *n.s.* not significant

On the other hand, vitrification, which is a progressive physiological problem, requires careful monitoring due to its gradual development with detrimental effects on the cultured plants. In a recent comprehensive review by Polivanova and Bedarev (2022), various factors contributing to vitrification were elucidated. The review highlighted that high humidity, insufficient lighting, elevated sugar levels, and excessive nitrates in in vitro culture conditions escalate the likelihood of vitrification. Therefore, as the duration of in vitro culture extends and plants are exposed to these conditions for a longer period, the severity and frequency of vitrification can increase. Secondly, the reduced ability of plants to adapt to this condition is a contributing factor to the progression of vitrification. In plants exposed to vitrification, the disruptions in cellular structures and physiological processes become more pronounced over time. Lastly, considering vitrification as a progressive problem, it can lead to increased challenges during acclimatization process of plants. Plants exposed to vitrification may struggle to adapt to lower humidity levels, and their survival rates in low humidity conditions during acclimatization can decrease.

Most of these factors were associated with the data presented in this study, which is the first of its kind conducted on *A. melanocarpa*. The findings clearly demonstrate that a protocol aimed at increasing shoot multiplication may inadvertently lead to vitrification. As a result, this report unveils, for the first time, a critical period of 20 days during which no vitrification occurs. However, it then suggests that certain media compositions may promote such abnormalities (see from 23rd day onward in Table 1). None of the earlier reports on clonal propagation of *Aronia* species mention this complex phenomenon in a long-term

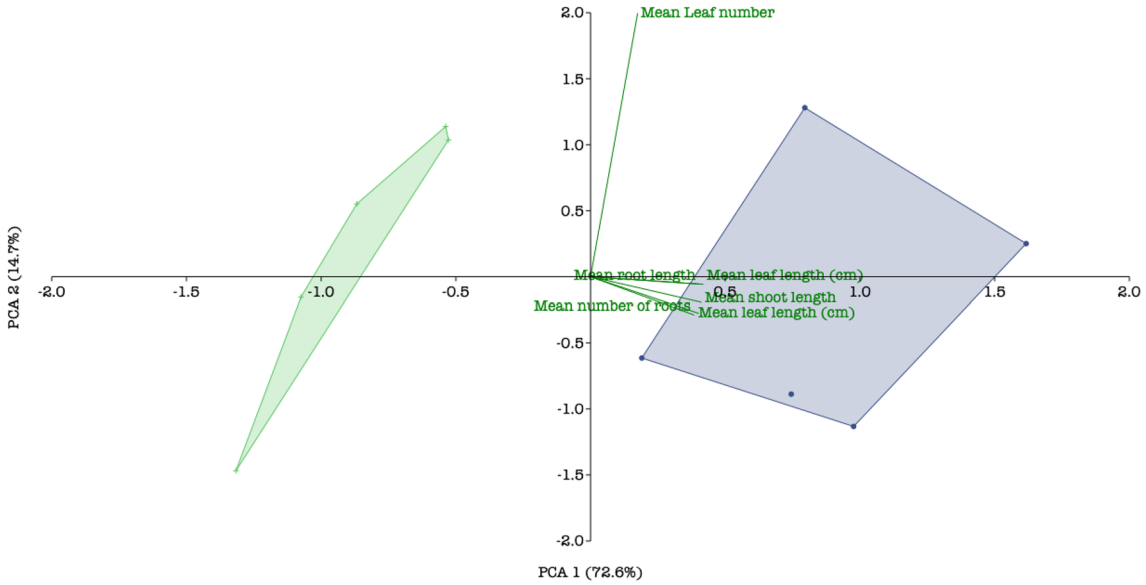

Fig. 5 Principal component analysis of vitrified (*green area*) and recovered (purple area) *Aronia melanocarpa* clones after 20 days of acclimatization. The percentage of variance reflects the proportion of total variance of the mean leaf and root number, mean root, shoot and leaf length, explained by each principal component

cultivation although they reported successful regeneration systems. For example, Rusea et al. (2018) reported that the highest shoot regeneration of *Aronia* was observed with a combination of 5.0 mg/L BAP and 0.5 mg/L IBA in MS medium. However, here we clearly showed that the highest BAP concentration and its interaction with sucrose (3%) was also conducive to vitrification over time. Although this period reduces the number of shoots in certain recovery media, it also enhances the recovery rate of vitrified plants in vitro, consequently increasing their survival rate during acclimatization. The literature on clonal propagation in vitro for a wide variety of crops is vast. While these studies acknowledge the existence of vitrification as a potential issue, many of them do not extensively report on vitrification when it comes to long-term production-oriented in vitro systems intended for commercial purposes. Instead, they generally mention vitrification as a physiological disorder that can be addressed by adjusting the composition of the growth medium (Toaima et al. 2016) or improving storage conditions, such as providing adequate light and air ventilation to the culture vessels (Casanova et al. 2008; Saez et al. 2012) In this study, it was demonstrated that sucrose concentration played a critical role in shoot growth. Higher sucrose concentration (3%) which is actually optimal for almost all tissue culture systems, generally resulted in increased shoot numbers. The optimal shoot number intervening the vitrification was observed with 3% sucrose and 0.5 mg/L BAP combination (M12 in Fig. 1), suggesting the positive influence of these conditions on shoot multiplication. In other words, normal initial tissue organization can slow down the vitrification process. Ensuring that the tissue is properly organized and differentiated before the onset of vitrification may help reduce its severity. However, 3% sucrose, combined with higher BAP levels (1.0- or 2.5 mg/L) led to an increase in vitrification over time. This indicates that an optimal balance between sucrose and BAP concentrations might be crucial for successful shoot multiplication without promoting vitrification. In contrast to our findings, Toaima et al. (2016) reported the micropropagation and vitrification of *Gypsophila paniculata*, where 0.5 mg/L each of BA and NAA in MS medium with higher sugar levels (40–60 g/L) had an inhibitory effect on shoot formation, shoot number, shoot length, and reduced the rate of vitrification. They also mentioned that the high sucrose amounts could disrupt cell development, limit nutrient uptake, induce osmotic stress, and act as a stress agent in vitro. Conversely, a low sucrose concentration promoted vitrification due to increased water availability. Different genotypes may exhibit variations in their physiological and morphological characteristics, as well as their responses to similar growth regulators and media compositions (Polivanova and Bedarev 2022). However, in our study, MS media with 3% sucrose concentration showed an increase in the vitrification ratio over time. As the sucrose concentration increased, the vitrification ratio also increased, resulting in a positive correlation between sucrose levels and vitrification. Secondly, lower sucrose concentrations (1% and 2%) did not initially exhibit vitrification until a certain point in the observation period. However, by the 38th

day, the vitrification ratio increased significantly on MS medium containing 2% sucrose plus 5.0 mg/L BAP and 3% sucrose plus 1.0 mg/L BAP. Even lower sucrose and BAP levels can contribute to vitrification over time (e.g. 1% sucrose plus 0.5 mg/L BAP after 65 days of cultivation in Table 1). Thirdly, the specific combinations of sucrose and BAP concentrations in the media influenced vitrification as well. Higher concentrations of BAP, particularly when combined with higher sucrose levels, resulted in increased vitrification ratios. Thus, sucrose concentration should be carefully controlled to optimize shoot regeneration without promoting vitrification. In this sense, further research is needed to better understand the physiological mechanisms underlying this phenomenon. Several studies have observed a correlation between cytokinin concentrations and the degree of vitrification in different plant species, including aloe, chlorophytum, garlic, gerbera, carnation and melon (Leshem et al. 1988; Sharma and Mohan 2006; Ivanova and Van Staden 2011; Liu et al. 2017; Gantait and Mahanta 2022). Based on these reports, reducing the concentration of cytokinins and auxins in the growth media is thought to be the best technique for preventing vitrification in in vitro cultures. Leshem et al. (1988) reported differences in vitrification between melon and carnation, indicating that melon vitrification is a cumulative process, with increasing vitrification observed after subcultures. In contrast, carnation vitrification is stable and maintained over long periods of growth. Their study also suggested that growth factors, such as excess cytokinin or auxin, could induce vitrification. Maintaining a proper balance of hormones may potentially reduce vitrification. Our findings align with these observations and are supported by correlation data, which highlight the impact of sucrose and BAP concentrations on callus size and shoot number. Additionally, we observed that *Aronia* vitrification does not follow a cumulative process but rather a progressive and stable pattern, depending on the duration of explant exposure to growth conditions. In this respect, Leshem et al. (1988) also reported that high concentrations of cytokinins could enhance ethylene biosynthesis, leading to vitrification. Therefore, controlling ethylene levels, possibly by using ethylene inhibitors or regulating the culture conditions, may help reduce vitrification. Zdarska et al. (2013) reported that cytokinins (BAP) and ethylene have a cross-talk relationship in regulating the size of the root apical meristem (RAM) and cell elongation in the root. It is possible that the successful recovery of plants with well-rooted shoots could be attributed to either a low concentration of exogenously added BAP in the growth medium or the presence of endogenous BAP levels in plants during in vitro cultivation. The balanced interaction between cytokinins and ethylene may contribute to the promotion of root growth and development, leading to the favorable outcome observed in recovered plants during acclimatization as presented in our study.

As for the acclimatization process, our findings highlight a clear relationship between vitrification and stomata characteristics. As the level of vitrification increased, we observed a progressive rise in the mean number of stomata cells, accompanied by alterations in stomata width and length. This finding was also correlated with the study reported by Asayesh et al. (2017). They reported the significant increase in stomatal size, density and index on the leaves of in vitro walnut plants as compared to the greenhouse plants. They also further commented on the high osmotic potential in in vitro plants, which led to a disturbance in the ability of stomata to close when the leaf water content sharply decreased during desiccation in *ex vitro* conditions. Notably, vitrified stomata lack a closure mechanism in response to various stimuli, including darkness, exposure to carbon dioxide, abscisic acid, hypertonic solutions, and water deficiency (Polinova and Bedarev 2022). In this present study, although the mean number of leaves was similar between healthy and vitrified *Aronia* plants, the vitrified plants had shorter leaf length and narrower leaf width. They also had a lower mean number of roots and shorter root length compared to healthy plants. This finding regarding the shape of stomata was consistent with the observations by Jausoro et al. (2010). However, no difference in stomatal density was reported between normal and vitrified clones of *Handroanthus mattos* in their study. In the present study, the survival rate of vitrified plants was reduced, with a significant decline in survival at lower humidity levels as mentioned. This indicates that vitrified plants are more vulnerable to environmental conditions and have a diminished ability to withstand lower humidity levels after acclimatization.

Conclusion

This study demonstrated the importance of determining optimum conditions for clonal propagation in *Aronia* to minimize vitrification and obtain high-quality shoots. In this context, it has been revealed that MS media containing 1 mg/L BAP with 10 g/L and 20 g/L sucrose as well as MS media containing 30 g/L sucrose and 0.5 mg/L BAP, are the most suitable media in terms of shoot regeneration with a low vitrification level. Increasing the level of BAP to achieve higher shoot regeneration in a short period of time will also increase the risk of vitrification. Therefore, a "vitrification termination protocol" can be utilized to rescue vitrified plants and reduce economic losses. However, the implementation of this protocol, along with the recovery period for vitrified plants, will incur time costs in the production schedule. Additionally, when using a high concentration of BAP, it is essential to transfer the shoots

to a rooting medium at an appropriate time to ensure successful rooting. This approach will not only result in higher shoot production but also enable the production of shoots with a low vitrification rate by adhering to the predetermined time intervals. Researchers or producers aiming to perform clonal propagation in *Aronia* are advised to consider these two aspects and choose the appropriate production plan according to the production strategies. In studies involving plant production through tissue culture under in vitro conditions, vitrification has become a global issue, necessitating scientific research focused on reducing vitrification or rescuing vitrified plants. Apart from the crucial factors of sucrose, BAP, and relative humidity highlighted in this study during acclimatization, further investigations are recommended to better understand the mechanism of vitrification. These investigations should involve examining the effects of different environmental conditions, where the genetic makeup can be tested and its impact on vitrification assessed.

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Author contributions Authors (NB and BY) contributed to the study conception and design. Material preparation, data collection and analysis were performed by the authors.

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Data availability All data obtained in this study were exclusively collected for research purposes related to vitrification. Some of the data presented in this manuscript, specifically those included in Table 1; Fig. 1, and Table 3, were sourced from the author's (Nida Bayhan) recent Master's thesis. This thesis has been submitted for evaluation and is currently awaiting publication. Furthermore, the methods employed to investigate low- and high-vitrification phenomena in this study were developed specifically for this research and were not reliant on any prior research or established methodologies. They are described comprehensively for the first time within this manuscript. Please note that the data sourced from the Master's thesis may have restricted availability until the thesis is officially published or released, so interested parties may need to request access through the corresponding author.

Declarations

Competing interest The authors have no relevant financial or non-financial interests to disclose. There are no competing interests about the hypothesis and relevant research questions mentioned in the study among the authors.

Ethical approval The plant species utilized in this study, *Aronia melanocarpa*, was selected as a model plant and was not used for commercial purposes. The plant species is not applicable with national and/or local legislation. The submitted work has not been published elsewhere in any form or language.

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