Effects of Sucrose and 6-Benzylaminopurine Concentrations on Shoot Regeneration and Vitrification in Aronia melanocarpa: Insights for Plant Tissue Culture Systems

Nida Bayhan
Bolu Abant İzzet Baysal Üniversitesi: Bolu Abant Izzet Baysal Universitesi
Buhara Yucesan (ibuhara@yahoo.com)
Bolu Abant İzzet Baysal Üniversitesi: Bolu Abant Izzet Baysal Universitesi https://orcid.org/0000-0002-6786-3973

Research Article

Keywords: Aronia melanocarpa, plant tissue culture, vitrification, shoot regeneration, 6-benzylaminopurine (BAP), sucrose

Posted Date: October 5th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-3356567/v1

Version of Record: A version of this preprint was published at Plant Cell, Tissue and Organ Culture (PCTOC) on January 16th, 2024. See the published version at https://doi.org/10.1007/s11240-023-02652-x.

Abstract

This study focuses on investigating the effects of sucrose and 6-Benzylaminopurine (BAP) concentrations on shoot regeneration and the development of vitrification in *Aronia melanocarpa*. Initially, the presence of BAP, in combination with varying sucrose concentrations, leads to a substantial increase in shoot number. The highest shoot formation is observed in the 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 sucrose levels were at 3% combined with higher BAP concentrations (1.0 or 2.5 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 stoma characteristics, such as density and size, 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 without necrosis, while the addition of 0.5 mg/L BAP promoted shoot growth but potentially inhibited root development. 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 six weeks under controlled greenhouse conditions.

1. INTRODUCTION

Aronia is a plant species belonging to the genus *Aronia* in the Rosaceae family. Within the *Aronia* genus, there 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). *Aronia melanocarpa*, the black-fruited aronia, can grow up to 1.2–2.4 meters in length. Since the major propagation technique of the crop is micropropagation, a literature review has been conducted on 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% sigma agar, and different concentrations of BA (max. 2.5 mg/L) for shoot multiplication for 8 weeks. Successful shoot multiplication and rooting of both *Aronia* varieties were achieved, and their easy adaptation to external conditions 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, 2,4-D, and IBA on shoot regeneration of Nero, a black chokeberry variety of *Aronia* testing different concentrations of BAP (max. 10 mg/L), 2,4-D (max. 1 mg/L), and IBA (max. 1 mg/L) in various combinations. However, no evidence of vitrification, a physiological disorder that affects tissue culture plants, has been reported in these studies. 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 behind vitrification continue to be explained through various studies. 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 solve the problem of 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 ve Sangwan-Norreel 2012). It was reported that increasing agar from 6- to 12 g/L reduced vitrification but also decreased plant growth (Hakkaart ve Versluijs, 1983). To test 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 (Pasqualetto vd., 1988). In the study by Dillen and Buysens (1989) on Gypsophila paniculata plants, they observed the effect of different culture medium components on vitrification. They 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. This suggests that the absence of essential nutrients and high agar concentration contributed to the occurrence of vitrification. This complex phenomenon influenced by various factors needs to be optimized to improve the success of plant tissue culture techniques. In summary, this study aimed to investigate the effects of sucrose and BAP which is the most common 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 the cytokinin BAP and its interaction with sucrose in relation to vitrification in tissue culture systems, as well as its impact on 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.

2. MATERIALS AND METHODS

2.1. Selection of plant material

Nero variety of *Aronia melanocarpa* explants, were excised from two-year-old mature bushy trees in the fields. The explants consisted of trimmed branches with sprouting fresh shoots. Meristematic nodal explants were cut into pieces measuring 2.5 cm in length and were subjected to surface sterilization following the protocol described in Tigrel et al. (2022). Briefly, the fresh shoot explants, after sprouting from the nodes on each branch, were immersed in a commercial bleach solution containing < 5% sodium hypochlorite for 20 minutes, followed by a 1-minute dip in 70% ethanol (v/v). After rinsing the explants with autoclaved water, the nodal explants were transferred to the growth medium.

2.2. Preparation of shoot regeneration and vitrification induction media

In this study, instant use 4.4 gr/L MS medium with vitamins (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 determined (see from M1 to M15, in Fig. 1). MS medium was solidified using 3.5 gr/L Gelrite (Duchefa, The Netherland) at pH 5.7 prior to autoclaving at 1.2 atm/121 °C. Experiments were conducted in glass jars (330 ml) with semi-transparent plastic lids. Each experiment utilized nine explants, with three replicates and three explants per glass jar (a total of 180 explants). In vitro cultivation was established in a growth chamber with an illumination of 16h using white cool fluorescent lambs (Master TL5 HE 14W T5, Philips, The Netherland), emitting around 3000 lux on each shelf. Humidity was maintained between 25- and 35%, temperature was kept at 24 °C using an air conditioner. In vitro cultivation was performed for 65 days, during which various growth parameters such as callus and shoot induction, regeneration, and vitrification were observed.

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 for plant cultivation. For instance, 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 other hand, low vitrification was observed in leaves that were slightly inwardly curled or not curled at all, displaying a translucent/wet appearance and light green color (Fig. 3d, e and f).

To induce and confirm vitrification with microscopy, an additional 65 days of cultivation were conducted using selected growth media supplemented with various concentrations of sucrose (1-, 2 or 3%) and BAP (0.5-, 1- and 5.0 mg/L) combinations in a total of seven treatments (see Table 1). Vitrification ratios were recorded on the 20th, 23rd, 30th, 38th, 44th, and 65th days of cultivation. Microscopic observations were utilized to describe the vitrification pattern. For this purpose, 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 stoma 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. The recovery media 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

(see Table 3). The shoots were placed in jars as mentioned earlier. The subsequent growth and development of the shoots were observed over a period of 31 days.

2.3. 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 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 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 (totally 20 days for whole acclimatization). All plants were then transferred to pots in a growth chamber with a relatively low humidity level of 15–25%.

2.4. Statistics

In the study, the data was analyzed using PAS 4.03 (Hammer et al., 2001) Statistical Software. Correlation tests 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.

3. RESULTS

3.1. 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 shoots (see M1, M6 or M11in Fig. 1). 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 corresponding sucrose concentrations. The highest shoot number was observed on MS medium in combination with 3% sucrose and 5.0 mg/L BAP (M15). Gradual increase of sucrose concentration (from 1–3%) increased the shoot number significantly at 5.0 mg/L BAP. Correlation coefficient between Callus size and Shoot number was calculated as 0.65 (Fig. 2). This positive correlation 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 in 3% sucrose plus 5 mg/L BAP (12 mm in Fig. 1). Both shoot number and callus formation showed 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 of BAP (from 1 to 5 mg/L). Vitrification was a clear pattern in the presence of BAP.

3.2. Observation of vitrification on selected BAP and Sucrose concentrations

Table 1 presents the effects of seven different regeneration media on the vitrification ratio of explants at various observation times. On the 20th day of observation, all media did not exhibit any signs of vitrification at this early stage. As the observation time progressed, the results varied depending on the composition of the regeneration medium indicating that different combinations of BAP and sucrose concentrations have varying effects on vitrification rates. 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 mg/L leads to a slight increase in vitrification rates when sucrose was fixed at %2 (22% vitrification on 23th day). The highest BAP concentration (5 mg/L) significantly increases 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 BAP, 3% sucrose + 1.0- or 5.0 BAP in Table 1), while the remaining media were considered as inducing *low vitrification.*

3.3. Identification of vitrified samples on the leaves

The data presented in Table 2 shows the stoma characteristics in leaf samples with varying levels of vitrification. The leaf morphology, mean number of stoma cells, stoma width, and stoma length 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 earlier based on morphological observation by naked eyes, and further detailed in microscopic observations accordingly (Table 1). The results of microscopy indicate that as the level of vitrification increases, there is a significant difference in the mean number of stoma cells, stoma width, and stoma length (see Table 2). In leaf samples produced from MS medium containing 3% sucrose and 0.5 mg/L BAP, the mean number of stoma cells per mm^2 was 76.12, stoma width was 27.92 µm, and stoma length was 42.21 µm indicating no vitrification. Comparatively, for the low vitrification, the mean number of stoma cells significantly increased to 106.27, stoma width increased to 32.47 µm, and stoma 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 stoma cells significantly increased to 135.43 per mm^2 with a mean of stomatal width 35.52-, and length 45.13 µm, accordingly (Table 2).

3.4. 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 a predominant pattern for the first two growth media combinations (0 Sucrose plus 0- or 0.5 mg/L BAP, Table 3). However, for the other growth media combinations without BAP, the recovery percentages indicating relatively high level of recoveries (%96 or 100%) without any sigh of necrosis, vitrification and tissue damage at all. By Day 31, the presence of necrosis was not observed, the recovery percentages of vitrified plants ranged from 83.9–100%. MS with varying sucrose concentrations without BAP (*i.e.* 1–3% sucrose) affected significantly growth parameters. For instance, 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 recovery of vitrified plants into healthy ones with well-developed root system. In the presence of 0.5 mg/L BAP, the growth media combinations showed different results in terms of shoot number per explant, callus size but not root number. The combinations with 0.5 mg/L BAP and any sugar concentration generally showed lower mean numbers of roots compared to the combinations without BAP. 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 while potentially inhibiting root growth. The 1% sucrose and 0.5 mg/L BAP combination showed the highest mean number of shoots (5.0) and mean callus size (10.0 mm). The 2% sucrose and 0.5 mg/L BAP combination exhibits intermediate values for all growth parameters, while the 3% sucrose and 0.5 BAP combination shows the highest mean shoot size (5.5 cm) but percentage of recovery was the lowest at 31st day (Fig. 4a).

3.5. Effects of vitrification on physiological parameters during acclimatization

In this experiment vitrified plants after 65 days of incubation were transplanted into the pots (Fig. 4b-c). They had generally a high level of vitrification and data presented in 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 leaf number. Vitrified plants exhibit significantly shorter leaf length (1.0 cm) and narrower leaf width (0.7 cm) 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) compared to 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 shoot length (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 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 complete loss of survival (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).

4. DISCUSSION

Tissue culture systems offer the possibility of large-scale propagation, but they also come up with two major challenges: contamination and vitrification. Contamination is a problem that can be easily recognized and addressed promptly, but its impact can be devastating if it spreads throughout the culture system. Therefore, it is crucial for researchers working with tissue culture systems to take all necessary precautions before initiating in vitro cloning to prevent contamination. 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. Very recently Polivanova and Bedarev (2022), reported a comprehensive review on vitrification addressing several reasons how vitrification to become one of major problems. Firstly, factors such as high humidity, inadequate lighting, excessive sugars, and nitrates in in vitro culture conditions increase the risk 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 the 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 30th day onward in Table 1). None of the earlier reports on clonal propagation of *Aronia* species do not mention this complex phenomenon in a long-term cultivation although they reported successful regeneration systems. For example, Rusea et al. (2018) reported that the highest shoot regeneration of *Aronia* was observed with the combination of 5.0 mg/L BAP and 0.5 mg/L IBA in MS medium. However, in this present study we clearly shown that the

highest BAP concentration and its interaction with sucrose (3%) is of promising effect on 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. In the literature, there are numerous papers available on clonal propagation in vitro for a wide variety of crops. While these papers acknowledge the existence of vitrification as a potential issue, many of these studies 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 hyperhydricity of *G Gypsophila paniculata*, and they revealed that 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 continued to mention 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 hyperhydricity 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, suggesting a positive correlation between sucrose levels and vitrification. Secondly, lower sucrose concentrations (1% and 2%) initially did not 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 (i.e., see 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. 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 vitrification. Several studies have observed a correlation between cytokinin concentrations and the degree of hyperhydricity 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 hyperhydricity 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 in our experiments 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 outcomes 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 stoma characteristics. As the level of vitrification increased, we observed a progressive rise in the mean number of stoma cells, accompanied by alterations in stoma width and length. Notably, hyperhydric 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 made 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.

5. 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.

Declarations

Author contributions

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

Acknowledgments

The authors would like to express their gratitude to the plant tissue culture companies, Xplant Co. in Istanbul and Zerafet Çiçekçilik Inc. in Konya, Türkiye for providing the plant material and for their valuable insights regarding the vitrification problem. This study was financially supported by the Scientific Council of Bolu Abant Izzet Baysal University under the project number 2022.10.04.1558. The authors would also like to extend their sincere appreciation to Prof. Dr. Goksel Özer for his assistance and expertise in conducting the microscopy observations.

Compliance with Ethical Standards

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.

Funding

This study was financially supported by the Scientific Council of Bolu Abant Izzet Baysal University under the project number 2022.10.04.1558.

Competing Interests

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.

Data Availability statements

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, Figure 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.

References

1. Bonga JM, Von Aderkas P (1992) In vitro culture of trees. Kluwer Academic Publisher
2. Brand MH, Cullina WG (1992) Micropropagation of red and black chokeberry (*Aronia* spp.). HortScience 27(1):81
3. Casanova E, Moysset L, Trillas MI (2008) Effects of agar concentration and vessel closure on the organogenesis and hyperhydricity of adventitious carnation shoots. Biology Plant 52:1–8
4. Çelebi-Toprak F, Alan AR (2018) A successful micropropagation protocol for three aronia (*Aronia melanocarpa*) cultivars. Acta Hortic 1285:173-176. https://doi.org/10.17660/ActaHortic.2020.1285.27
5. Dillen W, Buysens S (1989) A simple technique to overcome vitrification in *Gypsophila paniculata* L. Plant Cell Tiss Organ Cult 19:181–188 https://doi.org/10.1007/BF00043345
6. Gantait S, Mahanta M (2022) Hyperhydricity-induced changes among in vitro regenerants of Gerbera. S Afr J Bot 149:496–501 https://doi.org/10.1016/j.sajb.2022.06.038
7. Gribble K (1999) The influence of relative humidity on vitrification, growth and morphology of *Gypsophila paniculata* L. Plant Growth Regul 27:181–190 https://doi.org/10.1023/A:1006235229848

8. Hakkaart FA, Versluijs Joke MA (1983) Some factors affecting glassiness in carnation meristem tip cultures. Neth J Plant Pathol 89:47–53. https://doi.org/10.1007/BF01974443
9. Hammer Ø, Harper DAT, Ryan PD (2001) PAST: Paleontological Statistics software package for education and data analysis. Palaeontol Electron 4(1):9.
10. Huang LC, Kohashi C, Vangundy R, Murashige T (1995) Effects of common components on hardness of culture media prepared with gelrite™. In Vitro Cell Dev Biol Plant 31(2):84-89 https://doi.org/10.1007/BF02632242
11. Ivanova M, Van Staden J (2011) Influence of gelling agent and cytokinins on the control of hyperhydricity in Aloe polyphylla. Plant Cell Tiss Organ Cult 104:13–21. https://doi.org/10.1007/s11240-010-9794-5
12. Jausoro V, Llorente BE, Apóstolo NM (2010) Structural differences between hyperhydric and normal in vitro shoots of *Handroanthus impetiginosus* (Mart. ex-DC) Mattos (Bignoniaceae). Plant Cell Tiss Organ Cult 101:183–191 https://doi.org/10.1007/s11240-010-9675-y
13. Joyce SM, Cassells AC, Mohan Jain S (2003) Stress and aberrant phenotypes in vitro culture. Plant Cell Tissue and Organ Cult 74(2):103–121 https://doi.org/10.1023/A:1023911927116
14. Kataeva NV, Alexandrova IG, Butenko RG, Dragavtceva EV (1991) Effect of applied and internal hormones on vitrification and apical necrosis of different plants cultured in vitro. Plant Cell Tiss Organ Cult 27:149-154. https://doi.org/10.1007/BF00041283
15. Leshem B, Werker E, Shalev DP (1988) The effect of cytokinins on vitrification in melon and carnation. Ann Bot 62(3):271–276. https://doi.org/10.1093/oxfordjournals.aob.a087658
16. Liu M, Jiang F, Kong X, Tian J, Wu Z, Wu Z (2017) Effects of multiple factors on hyperhydricity of *Allium sativum* L. Sci Hortic 217:285–296 https://doi.org/10.1016/j.scienta.2017.02.010
17. Pasqualetto PL, Zimmerman RH, Fordham I (1988) The influence of cation and gelling agent concentrations on vitrification of apple cultivars in vitro. Plant Cell Tiss Organ Cult 14:31–40 https://doi.org/10.1007/BF00029573
18. Pierik RLM (1997) In vitro culture of higher plants. Springer Science and Business Media 1-160
19. Polivanova OB, Bedarev VA (2022) Hyperhydricity in plant tissue culture. Plants 11(23):3313. https://doi.org/10.3390/plants11233313
20. Rodríguez R, Tamés RS, Durzan DJ (Eds.) (2012) Plant aging: basic and applied approaches (Vol. 186). Springer Science and Business Media
21. Rusea I, Popescu A, Isac V, Șuțan AN, Hoza D (2018) Adventitious shoot regeneration from petiole explants in black chokeberry (Aronia melanocarpa). Scientific Papers Series B Horti 62:83-91
22. Saez PL, Bravo LA, Latsague MI, Sanchez ME, Rios DG (2012) Increased light intensity during in vitro culture improves water loss control and photosynthetic performance of *Castanea sativa* grown in ventilated vessels. Sci Hortic 138:7–16
23. Sangwan RS, Sangwan-Norreel BS (Eds.) (2012) The Impact of Biotechnology on Agriculture: Proceedings of the International Conference: “The Meeting Point Between Fundamental and Applied

in Vitro Culture Research," Held at Amiens (France), July 10–12, 1989 (Vol. 8). Springer Science and Business Media

24. Sharma U, Mohan JSS (2006) Reduction of vitrification in in vitro raised shoots of Chlorophytum borivilianum Sant. & Fernand., a rare potent medicinal herb. Indian J Exp Biol 44:499–505
25. Šnebergrová J, Čížková H, Neradová E, Kapci B, Rajchl A, Voldřich M (2014) Variability of characteristic components of aronia. Czech J Food Sci 32(1):25–30 https://doi.org/10.17221/540/2012-CJFS
26. Szopa A, Ekiert H (2014) Production of biologically active phenolic acids in Aronia melanocarpa (Michx.) Elliott in vitro cultures cultivated on different variants of the Murashige and Skoog medium. Plant Growth Regul 72(1):51–58 https://doi.org/10.1007/s10725-013-9835-2
27. Tigrel A, Arslan M, Arıcı B, Yücesan B (2022) Selection and preparation of explants for the clonal propagation of horticultural plants in plant factory systems. In: Gupta S, Chaturvedi P (eds) Commercial Scale Tissue Culture for Horticulture and Plantation Crops. Springer, Singapore. https://doi.org/10.1007/978-981-19-0055-6_2
28. Toaima N, Bosila H, El-Ateeq AA (2016) In vitro growth regulators, gelling agents and sucrose levels affect micropropagation of *Gypsophila paniculata* L. Middle East J Agric Res 5:313–323.
29. Ždárská M, Zatloukalová P, Benítez M, Sedo O et al. (2013) Proteome analysis in Arabidopsis reveals shoot- and root-specific targets of cytokinin action and differential regulation of hormonal homeostasis, Plant Physiology, 161(2): 918–930 https://doi.org/10.1104/pp.112.202853
30. Zimmerman TW, Rogers S, Cobb BG (1991) Controlling vitrification of petunia in vitro. In Vitro Cell Dev Biol Plant 27(4):165–167 https://doi.org/10.1007/BF02632211
31. Ziv M, Meir G, Halevy A (1983) Factors influencing the production of hardened glaucous carnation plantlets in vitro. Plant Cell Tiss Organ Cult 2:55–65 https://doi.org/10.1007/BF00033553

Tables

Table 1. Vitrification ratios (ranging 0% to 77%) for each combination of media on the corresponding day. *Colors of the cells also sign the degree of vitrification.*

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*

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

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]

[i] *Mean separation within treatments indicated by different letters, by Tukey's post-hoc test at P≤0.05.*

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 (13^{th} and 31^{st} 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

[i] *Mean separation within treatments indicated by different letters, by Tukey's post-hoc test at P≤0.05. Sucrose concentration is % (w/v), BAPis mg/L.*

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±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%

[i] *Significant difference at * P≤0.01, **P≤0.001, *** P≤0.0001; n.s.: not significant*

Figures

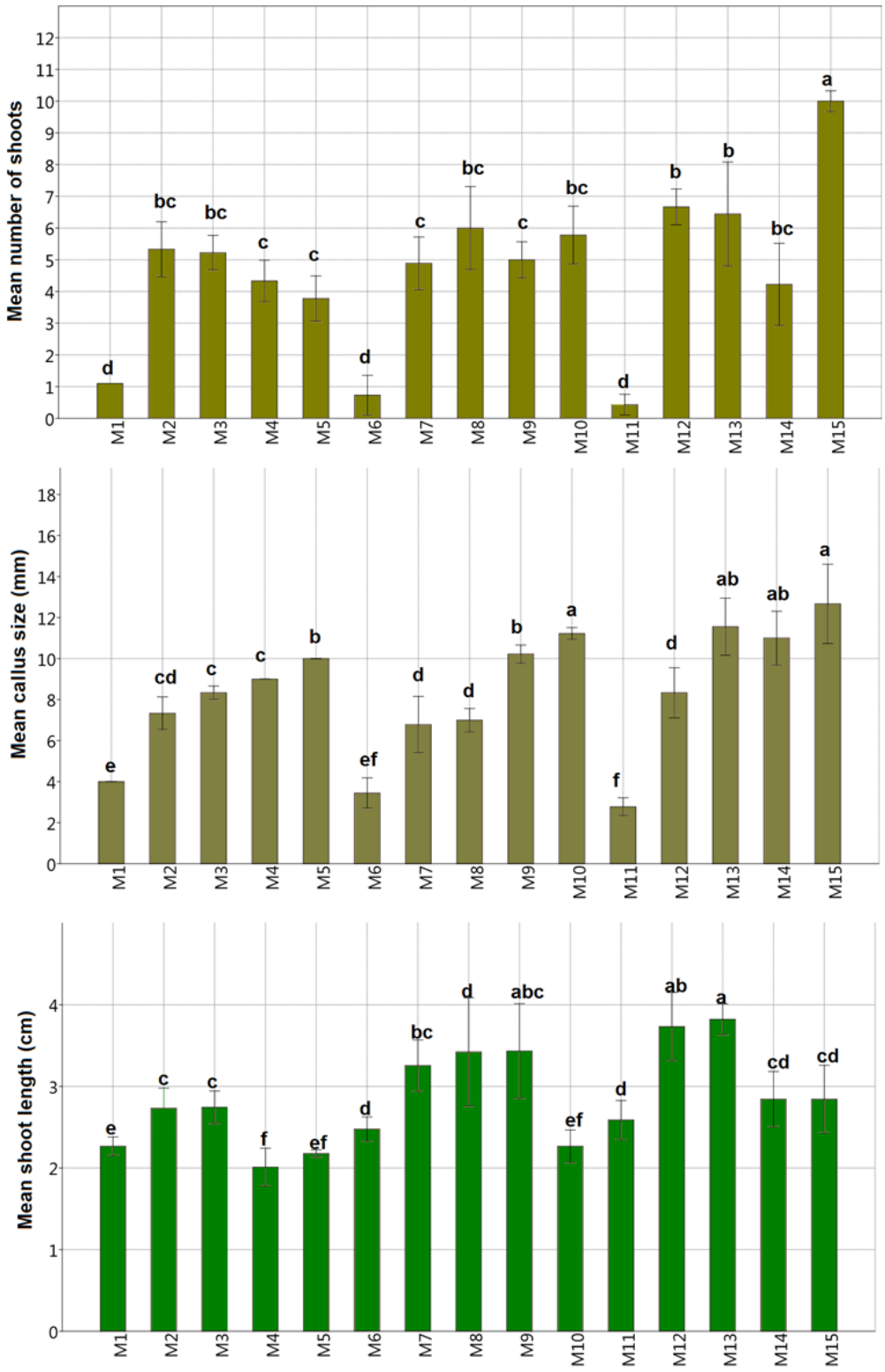

Figure 1

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.*

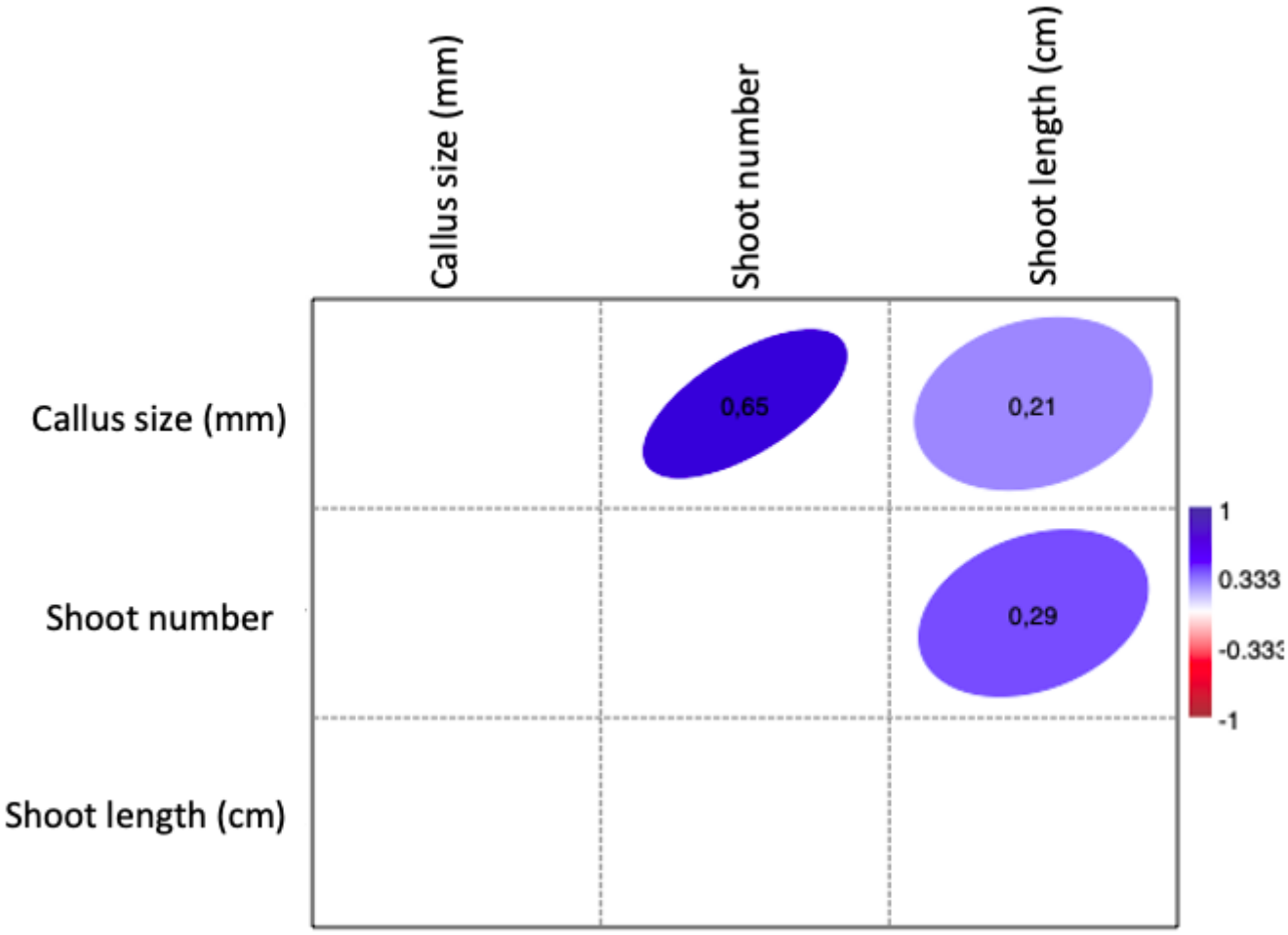

Figure 2

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

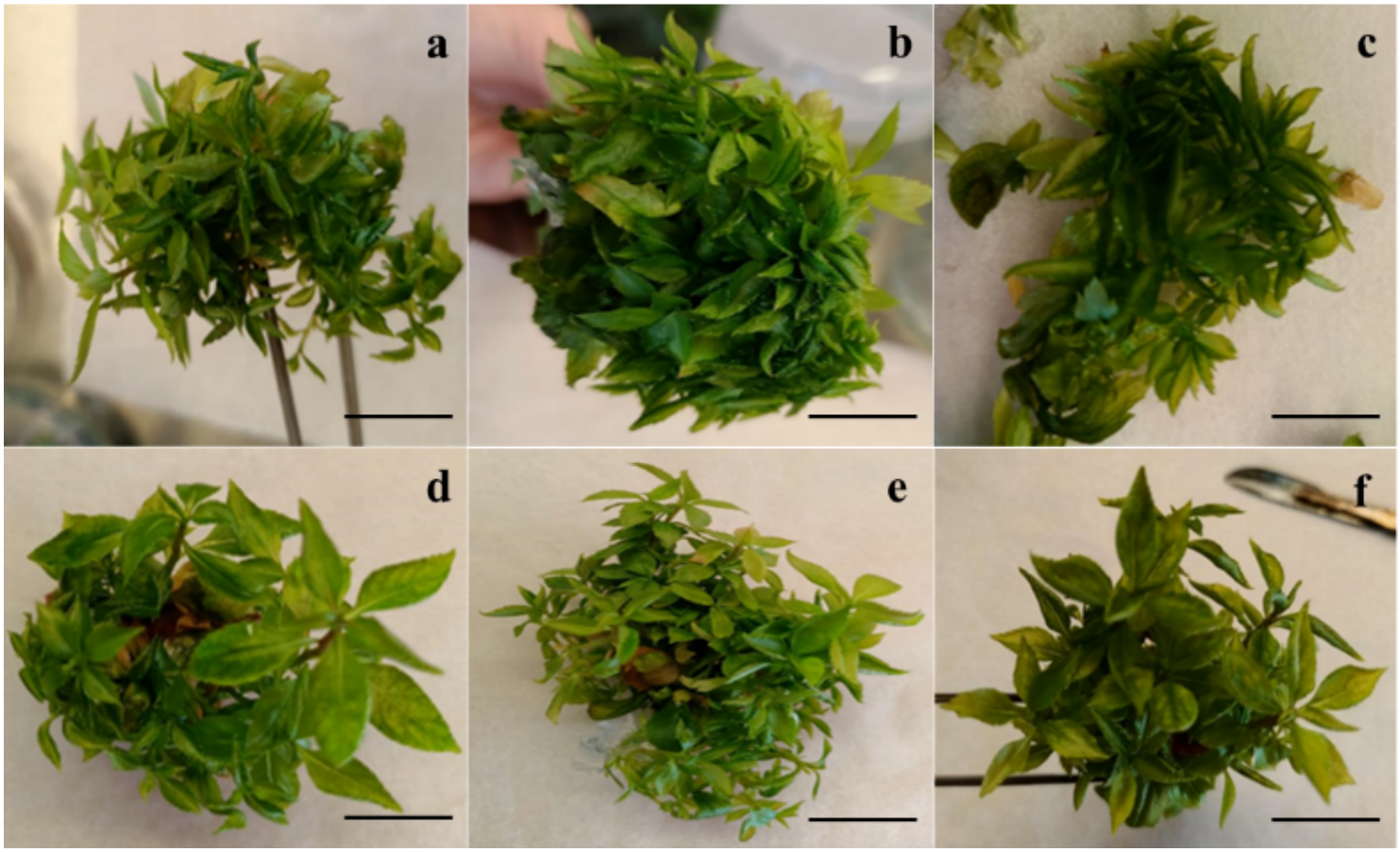

Figure 3

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

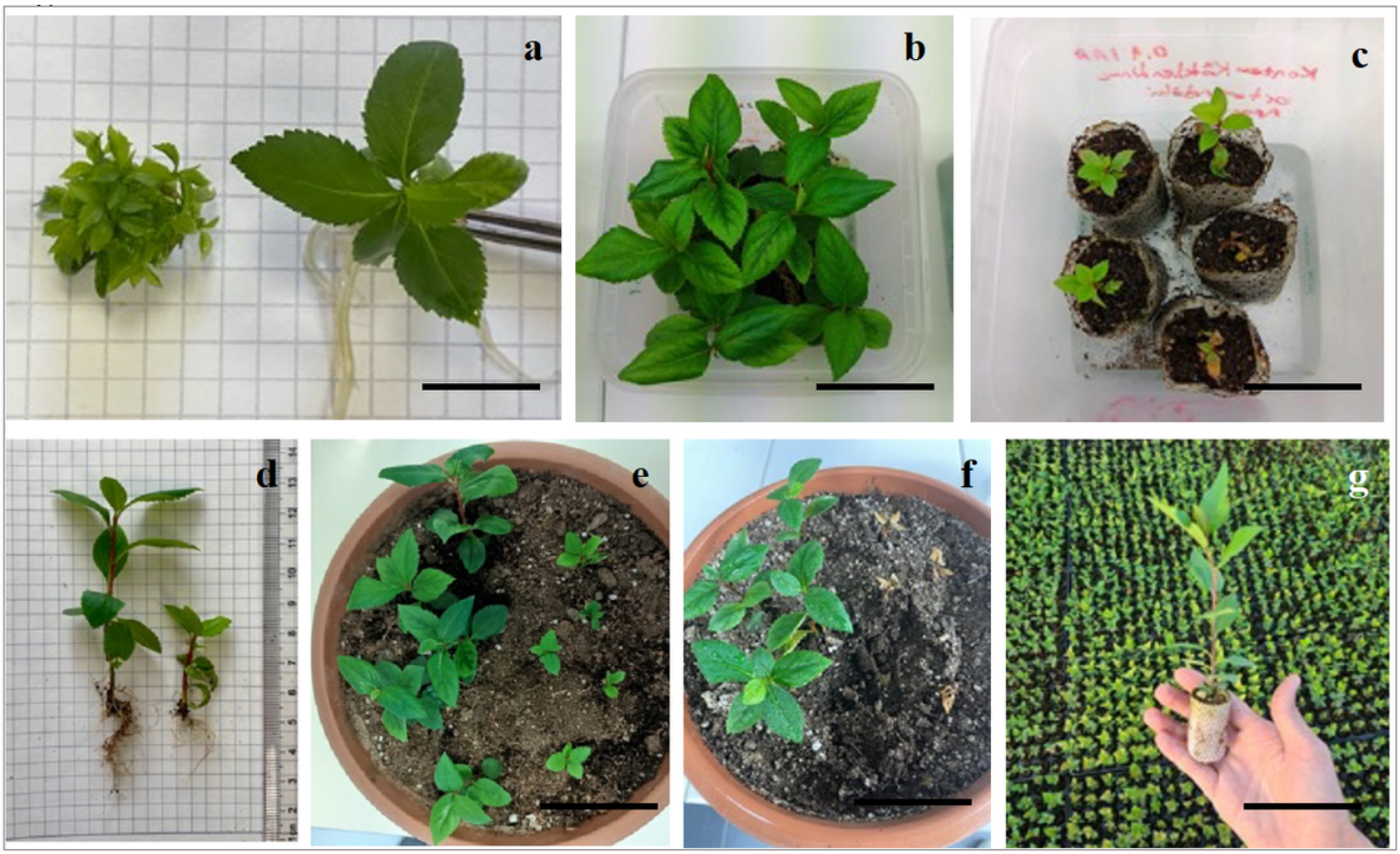

Figure 4

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), comparision 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).

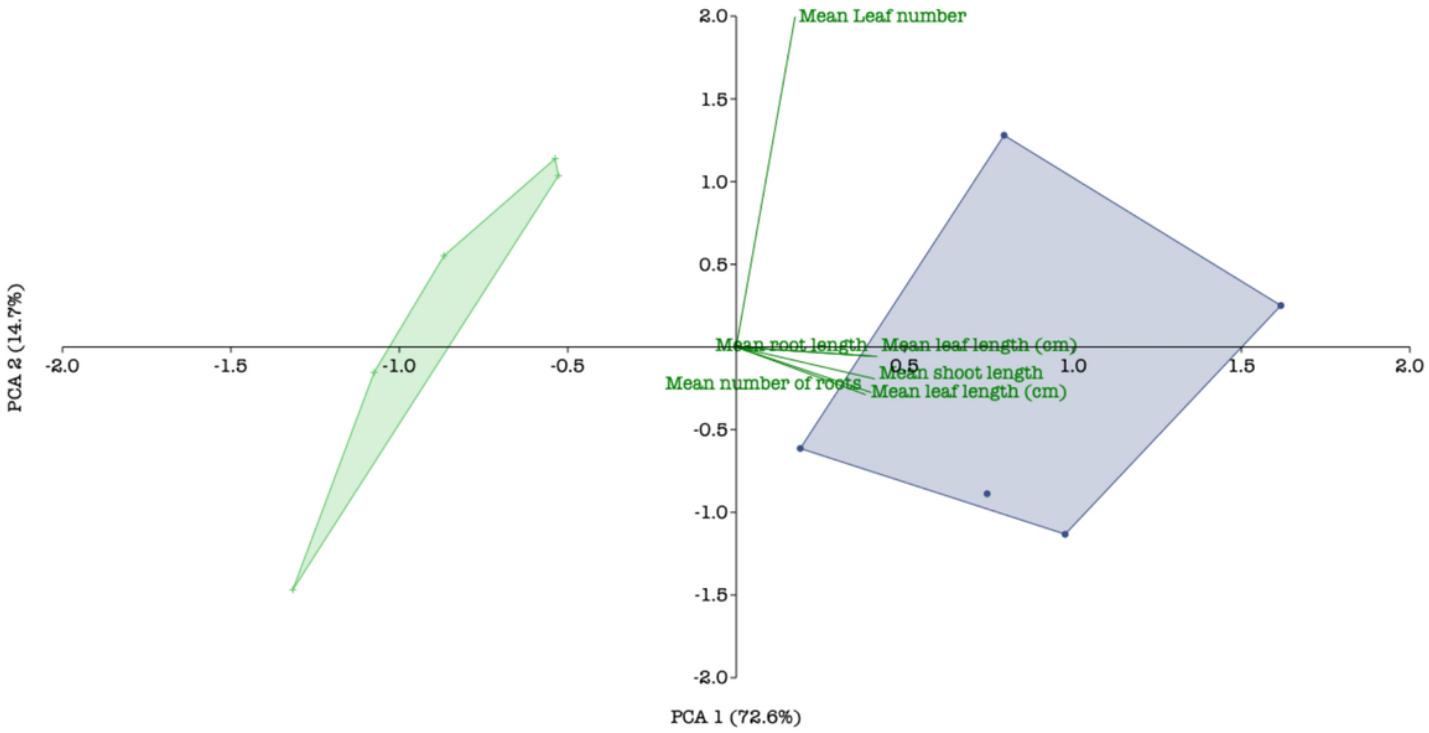

Figure 5

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 explained by each principal component*.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Suppl.Stomata.docx