



Somatic embryogenesis in *Vaccinium vitis-idaea* L.: a high-fidelity micropropagation approach producing true-to-type plants with enhanced phytochemical profiles

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Abstract

The “superfruit” lingonberry (*Vaccinium vitis-idaea* L.) is well known for its enormous health benefits and rich phytochemical contents. However, its regeneration and propagation face significant challenges due to the woody nature of the plant and poor proliferation rates using conventional methods. By using juvenile leaf explants of two *V. vitis-idaea* genotypes, this study introduces a novel and efficient somatic embryogenesis (SE) protocol for lingonberry micropropagation. The berry basal medium supplemented with 5.5 μM thidiazuron was optimized for the SE protocol where 92% embryogenic callus induction was achieved. The effectiveness of plantlet regeneration was greatly increased by employing 4.0 μM zeatin. Scanning electron microscopy study and histological analyses confirmed the development, maturation, and structural integrity of somatic embryos. Clonal fidelity study between the donor and regenerated plants by expressed sequence tag-simple sequence repeat (EST-SSR) and genomic simple sequence repeat (GSSR) ensured the genetic stability and maintenance of trueness-to-type of the SE micropropagules. The SE-regenerated plants showed higher levels of flavonoids, anthocyanins, and antioxidant activity than donor plants, indicating their improved nutritional and medicinal potential. Furthermore, SE-regenerated plants possessed elevated levels of antioxidant enzymes like catalase and superoxide dismutase, which enhanced their ability to withstand stress. The findings from this study provide a scalable, reliable, and sustainable approach to lingonberry micropropagation, combining genetic fidelity with enhanced phytochemical profiles.

Key message

A novel and optimized somatic embryogenesis protocol for *Vaccinium vitis-idaea* which ensured high clonal fidelity, enhanced phytochemical profiles, offered a scalable, genetically stable micropropagation method.

Keywords Somatic embryogenesis · Thidiazuron · Scanning electron microscopy · Histological analysis · Clonal fidelity · Phytochemical profiles

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Abbreviations

SE	Somatic embryogenesis
TDZ	Thidiazuron
ZEA	Zeatin
SJRDC	St. John's Research and Development Centre
BM	Berry basal medium
SEM	Scanning electron microscopy
EST-SSR	Expressed sequence tag simple sequence repeat
GSSR	Genomic simple sequence repeat
PEMs	Proembryogenic cell masses
SSE	Secondary somatic embryos
PSE	Primary somatic embryos
GSE	Globular somatic embryo
TPC	Total phenolic content
TFC	Total flavonoid content
TAA	Total antioxidant activity
ACN	Anthocyanin activity
CAT	Catalase
SOD	Superoxide dismutase
GR	Glutathione reductase

Introduction

The lingonberry (*Vaccinium vitis-idaea* L.) is one of the most economically important berry crops belonging to the *Ericaceae* family (subfamily: *Vaccinioideae*). It is widely distributed in various regions of North America, Iceland, Europe, and Asia (Debnath & Arigundam 2020). In North America, these berries are located north of the glacial barrier, ranging from northwest Greenland to New England through the Canadian Arctic. Lingonberries are small red berries that encompass more than 4250 species across 124 genera (Christenhusz & Byng 2016; Sikdar et al. 2022). The berries can be eaten in a variety of ways including raw or cooked in lingonberry jam, compote, candies, syrup, or juice. Lingonberries are referred to as "superfruits" due to their exceptional concentration of polyphenols and antioxidants such as vitamins A, C and E. Additionally, studies conducted in vivo and in vitro have suggested that lingonberries may have a variety of positive health impacts, including anti-inflammatory, antioxidant, and anticancer properties (Kowalska 2021). In vitro research utilizing a solvent like acetone showed neuroprotective benefits as well. Recent studies of Raudone (Raudone et al. 2019) have shown that leaf extracts of lingonberries are a promising source of bioactive chemicals with significant antioxidant activity and have excellent reducing, radical scavenging, and chelating capabilities (Vilkickyte et al. 2020).

Lingonberries, renowned for their remarkable health benefits, are increasingly in demand globally emphasizing

the need for efficient and sustainable propagation methods. Micropropagation techniques such as axillary shoot proliferation and adventitious shoot regeneration (Debnath and Arigundam 2020) using semi-solid and liquid media in bioreactor systems (Arigundam et al. 2020) have been extensively studied in lingonberry. These techniques showed greater effectiveness than the traditional methods of lingonberry propagation like rhizome division and stem cuttings (Debnath 2005) (Debnath and Arigundam 2020). Previously it was reported that using a bioreactor system (Debnath and Arigundam 2020), can lead to faster plant multiplication with higher yields. However, it has challenges like hyperhydricity (Arigundam et al. 2020), tissue lignification (Barros et al. 2015), and oxidative stress (Benson & Roubelakis-Angelakis 1994). Some significant challenges like genetic instability and somaclonal variation in conventional methods (Chavez-Cortazar et al. 2020) also affect large-scale propagation and long-term conservation. Thus, this led to the exploration of a new method renowned as somatic embryogenesis (SE) which aimed at achieving higher proliferation rates while overcoming the challenges faced by the previously used methods.

SE offers a revolutionary alternative to axillary shoot proliferation and adventitious shoot regeneration. From the studies of (Moradi et al. 2017) on *E. veratrifolia* it was shown that SE is a rapid vegetative propagation technique for conservation. Plant propagation through SE has several benefits over traditional micropropagation techniques (Bhattacharya et al. 2010), which entails the simultaneous formation of root and shoot meristems, eliminating the rooting step. SE enables clonal mass propagation, ensures genetic stability, and supports cryopreservation (Pais 2019). Moreover, SE is superior to shoot proliferation and shoot regeneration due to its higher propagation rates and production of true-to-type clones. Despite the success of SE explored in other woody plants like Babassu (palm tree) (Saleh et al. 2024), *Liriodendron* (Long et al. 2023), Cork oak (*Quercus suber* L.) (Ben Ali et al. 2023), Blueberry (Ghosh et al. 2018) it remains un-explored for lingonberries. During SE, somatic cells undergo both morphological and biochemical transformations, resulting in the development of structures such as globular, oblong, heart, torpedo, and cotyledonal shapes (Zimmerman 1993). Moreover, the research by Vahdati et al. (2004), highlights the potential of SE for mass propagation of Persian walnut, which is also a woody plant and emphasizes the need to optimize maturation conditions and the use of PGRs to enhance germination efficiency. Given the numerous advantages of somatic embryogenesis over conventional micropropagation methods, this research aims to fill the gap by investigating the use of SE as a micropropagation strategy for lingonberry.

Clonal stability is a critical factor in commercial micropropagation. While working with SE micropropagation, it is also important to produce and maintain true-to-type clones (Goyali et al. 2015), (Debnath 2011). Plants regenerated in vitro often face stress-induced changes, leading to somaclonal variation that may be genetic, epigenetic or both (Debnath & Ghosh 2022). Such variations, typically arising during callus formation from somatic cells, are heritable and undesirable for large-scale cultivation due to potential deviations from the desired traits of donor plants. To mitigate this, thorough screening of regenerated plants is essential to prevent unexpected variability and maintain genetic integrity. Although clonal fidelity can be assessed through morphological, biochemical, and physiological characteristics, DNA-based molecular markers are preferred for their reliability and immunity to environmental influences. Based on the studies by Sadat-Hosseini et al. (2019), somatic embryos have been identified as valuable targets for induced mutagenesis, cryopreservation, and germplasm conservation. They are also well known for transformation and gene editing to support plant improvement and functional gene analysis (Sadat-Hosseini et al. 2019). Therefore, the present study evaluated the clonal stability of regenerated plants using molecular markers expressed sequence tag—simple sequence repeat (EST-SSR) and genomic simple sequence repeat (GSSR) (Goyali et al. 2015).

Thus, this study aims to introduce SE as a novel micropropagation strategy for the first time in lingonberry. Electron microscopy and histological analysis were used to confirm the induction, formation, and maturation of SE plantlets. Genetic fidelity was verified using EST-SSR, and GSSR markers, ensuring true-to-type regeneration. Besides, comprehensive phytochemical profiling was done in SE-regenerated plants. This research establishes a scalable, efficient, and robust protocol for lingonberry propagation, marking a significant advancement in the cultivation of this superfruit.

Material and methods

Plant materials and culture condition

For the experiments, two lingonberry genotypes were used: the cultivar Erntedank (Cv1) and a hybrid designated as "H1" (identity undisclosed), developed at the St. John's Research and Development Centre (SJRDC), Agriculture and Agri-Food Canada, St. John's, Newfoundland and Labrador, Canada. These plants comply with institutional, national, and international guidelines and legislation. The genotypes Cv1 and H1 served as donor plants, maintained in a greenhouse (designated as Cv1-G and H1-G) at SJRDC. They were

grown in black plastic pots (10.5 × 10.5 × 12.5 cm) containing a 3:1 peat-to-perlite mixture (v/v) under natural light (maximum 90 $\mu\text{mol m}^{-2}\text{s}^{-1}$ photosynthetic photon flux) at 20 ± 2 °C and 85% humidity (Debnath 2017). Dr. Samir C. Debnath, the corresponding author, supervised the research and conducted the formal identification of the donor plants.

Somatic embryo induction and maturation

Juvenile leaves, around two to three weeks old, were obtained from donor plants, maintained in greenhouse. The leaves were carefully surface sterilized in 15% commercial bleach solution (0.79% NaOCl, 0.1% Tween 20) for 20 min, followed by a quick 70% ethanol and three sterile water rinses (Debnath 2009). The leaves were then gently scraped or slightly cut using a sterilized blade. Immediately after cutting, for embryo induction the leaves were placed on berry basal medium (BM) containing three-quarter micro salts and macro salts of Debnath and McRae (2001) supplemented with (per liter) 4% (w/v) sucrose, 0.6 g/L of Gelrite, and 1.4 g/L of agar (Sigma Chemical Co.). A total of 4 leaves were placed on a 100 mm × 25 mm Petri dish with a clear lid (Fisher Scientific, Fair Lawn, NJ, USA) containing 25 ml of BM. Leaves were positioned with their lower surface facing the medium. Thus, each petri plate with different concentrations of TDZ contained 4 leaf samples, with 3 replications of each concentration.

For the induction of somatic embryos, various concentrations (μM) of thidiazuron (TDZ) (2, 5.5, 8.5, 10, and 0 as the control) were used. To prevent contamination, the Petri dishes were covered with a layer of parafilm to seal the edges. The medium's pH was adjusted to 5.0 and TDZ concentrations were freshly prepared and added in the medium before autoclaving the dishes at 121 °C for 20 min (Ghosh et al. 2018). Each treatment was replicated three times. The Petri dishes were then kept in the dark at 20 ± 2 °C for four weeks, followed by incubation under cool-white, fluorescent lamps providing diffused light (PPFD at 30 $\mu\text{mol quanta m}^{-2}\text{s}^{-1}$, 16 h photoperiod) (Ghosh et al. 2018) in similar culture conditions.

The somatic embryos of 9–10-week-old which developed with 2, 5.5, 8.5 and 10 μM TDZ, were transferred to Sigma bottles for embryo growth, plantlet elongation and finally regeneration to a new mature plant. The somatic embryos generated with a 2 μM TDZ concentration for genotypes Cv1 and H1 were labeled as Cv1-2 and H1-2 respectively. Similarly, embryos formed with concentrations of 5.5, 8.5, and 10 μM TDZ were denoted as Cv1-5.5, Cv1-8.5, Cv1-10 and H1-5.5, H1-8.5, H1-10, respectively for both genotypes. Somatic embryos were transferred containing 30 mL BM supplemented with 4.0 μM ZEA (Ghosh et al. 2018) for growth and formation of plantlets (no growth observed in

control Perti plates). Before autoclaving, all the PGRs were introduced to the medium. For the purpose of promoting shoot and root elongation, cultures were kept at 30 μmol quanta $\mu\text{mol m}^2/\text{s}$ under a 16 h photoperiod. Each treatment concentration consisted of three jars with five explants each. Three times the experiment was conducted (number of replicates for each was 3, thus $n=3$). After 8 weeks and again after 12 weeks of transfer, maturation of embryo and finally plantlet elongation were seen for the regenerated micropropagated somatic embryo regenerated plants in Sigma bottles.

Scanning electron microscope (SEM)

SEM plays crucial roles in the study of somatic embryogenesis. SEM holds distinct advantages that make it more suitable for certain aspects of proving somatic embryogenesis. Thus, embryogenic callus after 6–7 week of different stages were fixed in modified 2.5% glutaraldehyde (Oliveira et al. 2022) and 2% paraformaldehyde, pH-adjusted to 7.2 in 0.1 M phosphate buffer and was dehydrated for SEM analysis. Samples were fixed onto aluminum stubs and were gold coated. The produced samples were analyzed and captured using an MLA 650 FEG scanning electron microscope.

Histological analysis

Morphogenic structures that developed during the somatic embryogenesis of lingonberry genotypes underwent histological analysis. Callus samples from the respective genotypes were extracted from cultures and immersed in an FAA solution (formalin 37%, glacial acetic acid, and ethanol 70%, in a 1:1:18 v/v ratio) (Sharma & Millam 2004) for approximately 48-h at room temperature. The fixed plant samples were then processed using a Tissue-tech VIP-5 Tissue Processor to achieve critical drying and wax infiltration. The microtome used for cutting the blocks was Leica RM2135. The sections were then cut at 5 μm and placed on VWR Superfrost Plus slides, air dried and heated for 12 h at 60 °C. The processed tissues, oriented as needed, were embedded in wax within suitable metal cradles. Observation of the prepared slides was carried out using a stereomicroscope, and light micrograph imaging was performed utilizing a Lumenera Infinity camera.

Acclimatization

After 9–10 weeks of culture, maturation in treatments Cv1-2, Cv1-10, H1-2, and H1-10 was poor, with most embryos turning brown and few surviving. By 12 weeks, plantlet regeneration was minimal in these treatments. In contrast, healthy plantlets measuring 5–6 cm in length with 7–11 leaves were observed in Cv1-5.5, Cv1-8.5, H1-5.5,

and H1-8.5 treatments. Due to poor growth and maturation, plantlets from Cv1-2, Cv1-10, H1-2, and H1-10 were excluded from further study. Healthy, vigorous plantlets from Cv1-5.5, Cv1-8.5, H1-5.5, and H1-8.5 were carefully removed, agar residues were cleaned with sterilized water and used for subsequent experiments. Afterwards, they were planted in black plastic pots (25 $\times$ 18 $\times$ 6; East-Chem Inc. Mount Pearl, NL, Canada) containing a mixture of 3 parts peat to 1 part perlite (Ghosh et al. 2018) (v/v).

The potted plantlets were placed in a humidification chamber with a vaporizer, maintaining a temperature of 20 $\pm$ 2 °C, a 16 h photoperiod providing 55 μmol $\mu\text{mol m}^2/\text{s}$ of light, and a humidity level of 95% (Ghosh et al. 2018). Over two to three weeks, humidity was gradually reduced to 85%, a level suitable for the survival of somatic embryo regenerated plantlets in a growth chamber. In vitro-grown plantlets tend to transpire excessively due to improperly formed cuticles, epicuticles, and cuticular waxes, as well as delayed stomatal function. To prevent excessive water loss, micropropagated plantlets must be transitioned gradually from high to lower humidity. Once adapted, the plants were transplanted into 6-cm³ plastic pots filled with the same peat-perlite mixture and maintained in the growth chamber.

DNA extraction and clonal fidelity assessment using molecular markers

According to the method described earlier by Debnath (Debnath 2011) actively growing leaves (weighing between 100–110 mg per sample) were taken from SE regenerated plants (Cv1-5.5, Cv1-8.5, H1-5.5, H1-8.5) and donor plants (Cv1-G and H1-G). DNeasy Plant Mini Kits (Qiagen, 40,724 Hilden, Germany) was used to extract DNA from these leaf samples. The FastPrep-24 tissue and cell homogenizer (MP Biomedicals, Irvine, CA, USA) was employed to homogenize the leaf tissue. The manufacturer's instructions (Qiagen) were followed for DNA extraction. The genomic DNA was analyzed using agarose gel electrophoresis (1.8%) and its concentration was determined using an Ultrospec 2000 spectrophotometer. The A260/A280 nm ratio was used to assess the purity of the DNA. DNA with an A260/A280 ratio ranging from 1.8 to 2.1 was diluted to a concentration of 10 ng/ μL using 1 TE buffer, for subsequent amplification procedures.

The genetic fidelity of somatic embryo regenerated lingonberry plants was assessed using a total of 5 EST-SSR (prefixed CA or NA), AND 5 GSSR (prefixed VCC) primer pairs, as listed in Table 1. These primer pairs were synthesized by Integrated DNA Technologies (Coralville, IA). Goyali et al. (2015) utilized markers successfully with *Vaccinium* species to evaluate the genetic fidelity of micropropagated plants. The reaction mixture, consisting of 20 μL

Table 1 Primer names and respective sequence (F: forward, R: reverse), and annealing temperature, used to analyze the genetic fidelity in *V. vitis-idaea* in somatic embryo regenerated plants and donor plants

Primers	Primer sequence	Annealing temperature (°C)
<i>EST-SSR</i>		
CA23	F: GAG AGG GTT TCG AGG AGG AG R: GTT TAG AAA CGG GACTGTGAGACG	56°
NA741	F: CAA TCC ATT CCA AGC ATG TG R: GTT TGA TTT TGG GGG TTA AGT TTG C	60°
CA421	F: TCA AAT TCA AAG CTC AAA ATC AA R: GTT TAA GGA TGA TCC CGA AGC TCT	56°
<i>EST-SSR</i>		
NA824	F: AAA TCG TTG GTT TGG CTC TG R: GTT TGG GCC GAA AAG AAA TCG TAT	60.2°
NA41	F: TTC CTT TAG TCG CGT CAT CA R: GTT TAA GGT CGC TAC GAG ACT CCA	56°
<i>Genomic SSR</i>		
VCC_K4	F: CCT CCA CCC CAC TTT CAT TA R: GCA CAC AGG TCC AGT TTT TG	62°
VCC_S10	F: ATT TGG TGT GAA ACC CCT GA R: GTT TGC GGC TAT ATC CGT GTT TGT	60°
VCC_J1	F: CTC ATG GGT TCC CAT AGA CAA R: TGC AGT GAG GCA AAA GAT TG	58°
VCC_J9	F: GCG AAG AAC TTC CGT CAA AA R: GTG AGG GCA CAA AGC TCT C	60°
VCC_I2	F: AGG CGT TTT TGA GGC TAA CA R: TAA AAG TTC GGC TCG TTT GC	62°

in total, was composed of 2 µL of 25 ng of DNA template, 10 µL of Phusion green hot start II PCR master mix (high fidelity PCR master-mix) sourced from Thermo Scientific), 2 µL of both the forward and reverse primers, along with 7 µL of PCR-grade distilled water from Sigma Chemical Co. DNA amplification was conducted using a Mastercycler ep Gradient S (Eppendorf AG, 22,331 Hamburg, Germany) with a program consisting of an initial denaturation step at 94 °C for 10 min to facilitate a 'hot start'. The following cycling conditions were repeated 40 times: denaturation of the template DNA at 92 °C for 40 s, annealing of the primers at the appropriate temperatures (Table 1) for 70 s, and extension of the primers at 72 °C for 2 min. The final extension step involved a 10 min incubation at 72 °C. During the waiting period, the samples were stored at 4 °C. The annealing temperatures for all markers were standardized using the temperature gradient PCR method. Gel electrophoresis was performed using a 1.8% (w/v) agarose 3:1 HRB blend (High resolution; Amresco, Solon, OH, USA) gel to separate the amplified products. Gel casting was prepared using 4.3×Tris–borate EDTA (TBE) buffer and 0.5 µL 1×RedSafe™ (iNtRON biotechnology) nucleic acid dye. In addition to the amplified products, a 100-bp Low Ranger DNA ladder (Norgen Bioteck corp., Thorold, ON, Canada) were loaded onto the gel. The gel was run at 75 V for 1.5 h, and the DNA bands were digitally imaged using a gel documentation system (Gel Doc1000 Bio-Rad) under UV light. The DNA banding patterns were analyzed using an image analysis program (Gene Tools, Syngene).

The bands were categorized as present (1 band) or absent (0 band), representing a binary character. Reproducible bands were recorded after running the reactions at least twice.

Phytochemical analysis

Preparation of leaf samples for phytochemical profile assessment

Leaves from 12-week-old in-vitro SE regenerated plants (Cv1-5.5, Cv1-8.5, H1-5.5, H1-8.5) and young leaves from greenhouse maintained donor plants (Cv1-G and H1-G) were transformed into fine powder using liquid nitrogen and a mortar-pestle. Leaf powder, ranging from 200 to 500 mg, was mixed with a solution of 80% aqueous acetone containing 0.2% formic acid. This mixture was homogenized using a FastPrep-24 tissue and cell homogenizer (MP Biomedicals, Irvine, CA, USA). After shaking the homogenate for 30 min at 4 °C, it underwent centrifugation (Allegra 64R, Beckman Coulter Inc., Palo Alto, CA, USA) at 4 °C for 15 min at 13,000 g (Goyali et al. 2013). The resulting supernatant was collected as the extract. This extract was then used for quantifying total phenolic content (TPC), flavonoid content (TFC), and antioxidant activity (TAA).

For assessing enzymatic activity, particularly for antioxidant enzymes, leaf samples weighing 0.5 g were homogenized in an ice-cold buffer containing 50 mM Tris HCl (pH 7.5), 1 mM dithiothreitol (DTT), and 250 mM sucrose (Gao et al. 2014). The homogenates were prepared using

this buffer and then subjected to a 30 min centrifugation at 13,000 g at 4 °C. After centrifugation, the resulting supernatants were employed to conduct enzyme assays. Each of the biochemical analyses were conducted three times for every sample, and the mean values were utilized for subsequent statistical analysis.

Total phenolic content (TPC)

Folin-Ciocalteu reagent was used to determine the TPC from leaf extract following Goyali et al. (2013, 2015). Gallic acid was used as a standard and the data was calculated as milligrams of gallic acid equivalent per gram of fresh weight (mg GAE/g fw). The absorbance of both the gallic acid standard solution (5 mg/mL) and the leaf samples was assessed at a wavelength of 725 nm with the Ultrospec 4300 Pro UV–visible spectrophotometer, manufactured by Amersham Biosciences Corp. in San Francisco, CA, USA. The absorbance values were recorded in a linear manner using a standard calibration curve ($r^2=0.98$) that was established with the gallic acid standard solution (5 mg/mL; purity $\geq 98\%$) within the range of 2.5–10 $\mu\text{g/mL}$.

Total flavonoid content (TFC)

Colorimetric assay following the procedure described by Goyali et al. (2013, 2015) was used to determine TFC. Catechin was used as a standard and the data was calculated in milligrams of catechin equivalents per gram of fresh weight (mg CE/g fw). The absorbance was measured at 510 nm using an Ultrospec 4300 Pro UV–visible spectrophotometer (Amersham Biosciences Corp., San Francisco, CA, USA). A standard calibration curve was generated using a catechin solution (1 mg/mL) within the range of 20–200 $\mu\text{g/mL}$ ($r^2=0.99$).

Total antioxidant activity (TAA)

The scavenging activity against free radicals was assessed by determining the percentage inhibition of radicals. In this assay, 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was used as an artificially stabilized free radical. A portion of the diluted extract or gallic acid standard solution (5 mg/mL; $\geq 98\%$ purity) was added to 1.7 mL of a methanolic solution of DPPH (0.06 mM). The mixture was thoroughly mixed and kept in the dark at room temperature for 20 min. The absorbance of the mixture was then measured at 517 nm using an Ultrospec 4300 Pro UV–visible spectrophotometer. A blank was prepared using 80% aqueous acetone mixed with the DPPH solution. To establish a gallic acid standard curve, solutions with various concentrations ranging from 20 to 80 $\mu\text{g/mL}$ were prepared using gallic acid ($\geq 98\%$ purity).

The linearity of the gallic acid standard curve ($r^2=0.98$) was confirmed. The results were reported as milligrams of gallic acid equivalents (GAE) per gram of fresh weight (mg GAE/g fw) (Goyali et al. 2013, 2015).

Anthocyanin assay (ACN)

The quantification of monomeric ACN in the lingonberry extract was conducted using the pH-differential method (Goyali et al. 2013, 2015). To determine the ACN the absorbance was measured at 510 nm and 700 nm using an ultra-violet spectrophotometer. ACN was calculated using the provided formula:

$$\text{Anthocyanin content (mg/g)} = (A \times MV \times DF) / (\epsilon \times l)$$

where A represents the absorbance difference between pH 1.0 and pH 4.5 measurements at wavelengths λ_{510} and λ_{700} .

Additional variables used in the calculation are as follows:

MW (molecular weight)=449.2 g/mol for cyanidin-3-glucoside. DF represents the dilution factor, ϵ (epsilon)=26.9 mM extinction coefficients in $\text{g} \times \text{m/mol/cm}$ for cyanidin-3-glucoside. l represents the path length in centimeters.

The resulting value obtained from the calculation represents the total anthocyanin pigment concentration, expressed as cyanidin-3-glucoside equivalents (C_3 GE) in milligrams per gram of fresh weight.

Catalase (CAT) activity

For assessing CAT activity, the protocol from (Nardi et al. 2016) and (Verma & Mishra 2005) was followed. The activity was assessed by measuring the rate of hydrogen peroxide (H_2O_2) decomposition by catalase enzyme in the sample. The reduction in absorbance was observed at 240 nm over a 60 s period with 10-s intervals. Enzyme activity was calculated using the molar extinction coefficient of hydrogen peroxide ($43.6 \text{ M}^{-1} \text{ cm}^{-1}$) at 240 nm. The results were expressed as mmol/min/g of fresh weight.

Superoxide dismutase (SOD) activity

The SOD activity was evaluated using a procedure described by (Debnath et al. 2013). To start, 0.05 mol/L phosphate buffer at pH 7.8 (3 mL) was pre-chilled and combined with the powdered sample (0.3 g). After centrifuging the sample mixture at 4 °C for 10 min at 12,000 g, the supernatant was collected and used as the enzyme solution (this process was conducted on ice). In a test tube, a reaction mixture (2.95 mL) containing 130 $\mu\text{mol/L}$ Met, 750 $\mu\text{mol/L}$

NBT, 100 $\mu\text{mol/L}$ EDTA- Na_2 , 20 $\mu\text{mol/L}$ riboflavin, and 0.05 mol/L phosphate buffer at pH 7.8 was prepared, along with the enzyme solution (50 μL). The control group was treated with PBS (50 μL). After mixing thoroughly, one tube was kept in darkness, while the other tubes were exposed to light for 20 min. The amount of enzyme needed to decrease the rate of nitro blue tetrazolium reduction by 50% was defined as one unit of SOD activity (Samad et al. 2014).

$$\text{Scavenging activity (\%)} = 100 - [(A - B)/C] \times 100$$

where A is the absorbance of sample/standard with reagent; B is the absorbance of sample/standard without reagent; and C is the absorbance of the control.

Glutathione reductase (GR)

GR activity was determined at 340 nm for a duration of three minutes using the conversion rate from NADPH to NADP. In this procedure, 100 μL of the sample was combined with 900 μL of 50 mM HEPES buffer (pH 8.0), which contained 0.5 mM EDTA and 0.25 mM NADPH (Vyas et al. 2013). The reaction was initiated by introducing 1 mM GSSG, and the absorbance at 340 nm was recorded. To calculate the equivalent activity of GR is $\mu\text{mol/min/g}$ of fresh weight., a molar extinction coefficient of $6.2 \text{ mM}^{-1} \text{ cm}^{-1}$ was used.

Statistical analysis

The statistical software JMP Pro16 (Jones & Sall 2011) was used to perform the analysis of variance (ANOVA) and respective graphical analysis, for all characters. Each experiment was carried out using a completely randomized design with three replications, and the results were $P \leq 0.05$ evaluated (Ghosh et al. 2018). In terms of somatic embryo development, medium without PGR did not exhibit any response and was therefore left out of the analysis. The maturation of somatic embryos by TDZ 2 μM conc. and for 10 μM conc. was also determined to be ineffective in both genotypes, and the pertinent data were also removed from analysis. A crucial difference (P) of ≤ 0.05 was utilized to compare treatment means using Tukey's test. The outcomes are displayed as mean standard error. The relationships were significant at a 95% confidence interval ($\alpha = 0.05$).

Results

Somatic embryo induction, maturation, SEM and histological analysis

Somatic embryos at 9–10 weeks of age were developed in Petri dishes using different concentrations of TDZ (2, 5.5, 8.5, and 10 μM). After being placed in darkness for a period of 4 weeks, the initiation of globule formation at the edges of the leaf explant became evident (Fig. 1A). Subsequently, at 6–7 weeks, globular somatic embryos and heart shaped embryos emerged (Fig. 1B, C), accompanied by the early stages of cotyledonary somatic embryos on the apex of the globular mass. By the eighth week, under culture conditions, the observation shifted to the presence of somatic embryos with fused cotyledons in the form of SE cups (Fig. 1D). After 9 weeks, both somatic embryo development and maturation were observed. Notably, explants in petri dishes without thidiazuron (TDZ) showed no response in terms of embryo induction.

Scanning electron microscopy unveiled the progression of globular (Fig. 1E) and heart-shaped embryos (Fig. 1F). Along with SEM analysis, histological tests further validated the formation and maturation of somatic embryos in lingonberry. Formation of proembryogenic cell masses (PEMs) were observed (Fig. 1G). It also showed the clusters of secondary somatic embryos (SSE) formed from primary somatic embryos (PSE) (Fig. 1H), and formation of secondary globular somatic embryo (GSE) (Fig. 1I).

A significant interaction ($P \leq 0.05$) was observed between genotype types and TDZ concentrations in relation to the percentage of SE formation. 'Cv1-5.5' demonstrated the highest percentage of embryo formation (92%) across all TDZ concentrations, followed by 'Cv1-8.5' (67%), H1-5.5' (83%), H1-8.5' (75%), H1-10' (33%), Cv1-10' (25%), 'H1-2' (25%), and 'Cv1-2' (17%). The percentage of somatic embryo formation or percentage of somatic embryogenesis gives an overall success rate of the process in terms of how many explants were capable of producing embryos. It is important for evaluating the efficiency and effectiveness of somatic embryogenesis protocols in plant tissue culture. Notably, at 2 μM of TDZ, the percent embryo formation values were the lowest in both genotypes. The percentage of SE formation exhibited an upward trend with 5.5 and 8.5 μM concentrations of TDZ. ANOVA results indicated a significant impact of TDZ concentrations ($P \leq 0.05$) on the number of somatic embryos formed (Fig. 2A, B). At concentrations of 2 and 10 μM TDZ, the number of embryos per explant ranged from 2 to 7. However, at 5.5 and 8.5 μM TDZ, the number of embryos per explant varied from 21 to 17 in both genotypes. Therefore, in the present study for cultivar Cv1, the effect of TDZ concentration on somatic embryo

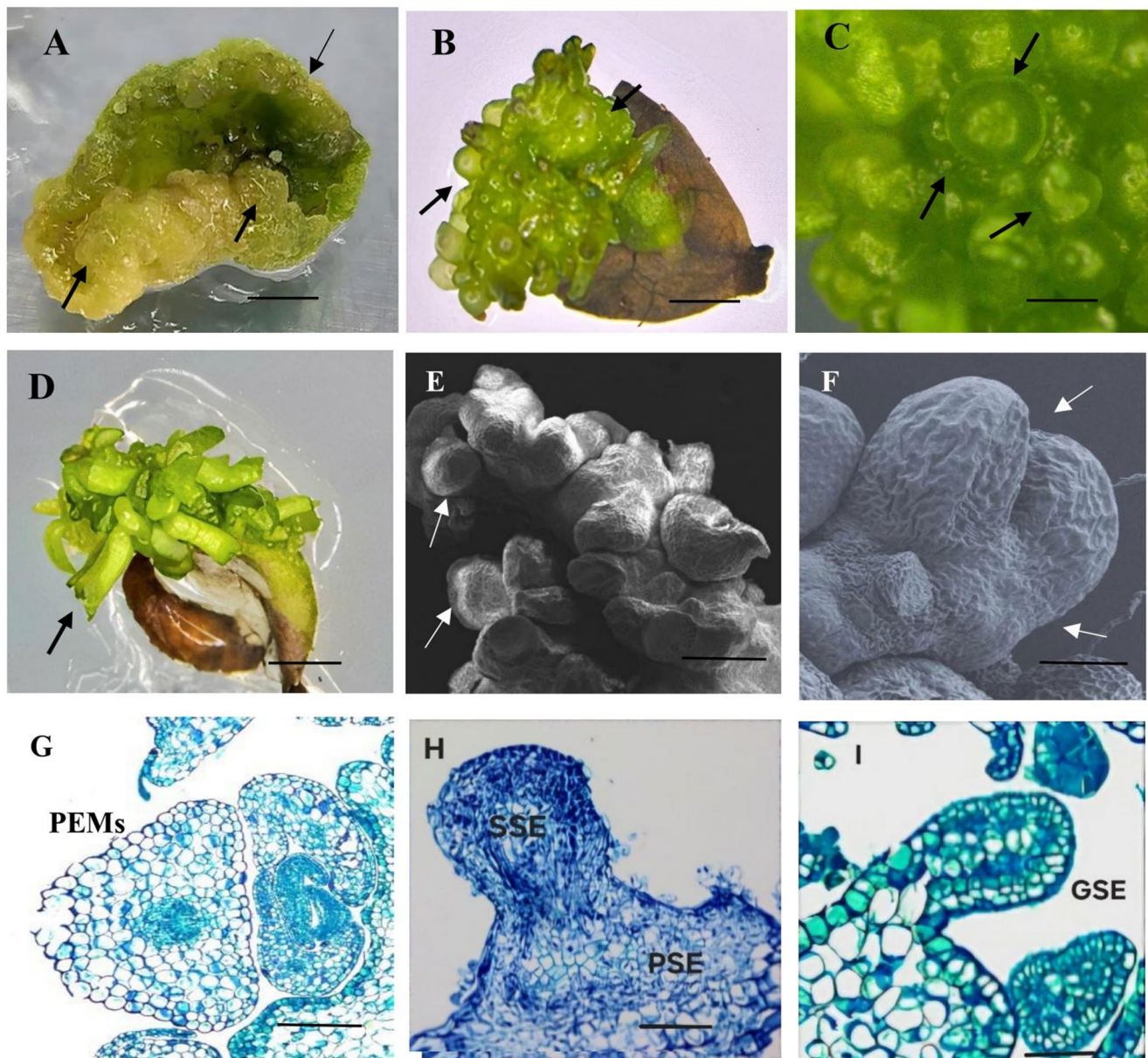


Fig. 1 Somatic embryogenesis from leaf explants of lingonberry. (A) shows the beginnings of the globules formation on the edges of leaf explant after 4 weeks (arrows), (B) shows formation of globular embryos and heart shaped embryos within 5–6 weeks of culture (arrows), (C) globular somatic embryo (arrows) (D) somatic embryo cup with fused cotyledons (arrows), (Bars=0.5 cm) (E) scanning

electron microscope showing globular somatic embryo, (F) heart shaped embryo development (arrows), (Bars=1 mm) (G) histological test showing formation of proembryogenic cell masses (PEMs), (H) clusters of secondary somatic embryos (SSE) formed from primary somatic embryos (PSE), (I) formation of secondary globular somatic embryo (GSE) (Bars=50 μ m)

formation was statistically significant (one-way ANOVA, $p=0.032$). Tukey's test showed that somatic embryo formation at 5.5 μ M TDZ was significantly higher compared to 2 μ M ($p=0.007$) and 10 μ M ($p=0.019$). Similarly, a significant difference was observed between 2 μ M and 8.5 μ M TDZ ($p=0.041$). However, no significant difference was observed between 2 μ M and 10 μ M ($p=0.732$), 5.5 μ M and 8.5 μ M ($p=0.273$), or 8.5 μ M and 10 μ M ($p=0.161$). Similarly for cultivar H1, one-way ANOVA showed statistically

significant effect of TDZ concentration on somatic embryo formation (ANOVA, $p=0.048$). Tukey's test showed that somatic embryo formation at 5.5 μ M TDZ was significantly higher than at 2 μ M TDZ ($p=0.038$). A similar significant increase was observed at 8.5 μ M TDZ compared to 2 μ M TDZ ($p=0.048$).

The results thus indicate that varying TDZ concentrations influenced embryo formation rates across different cultivars. The mature somatic embryos were transferred to a

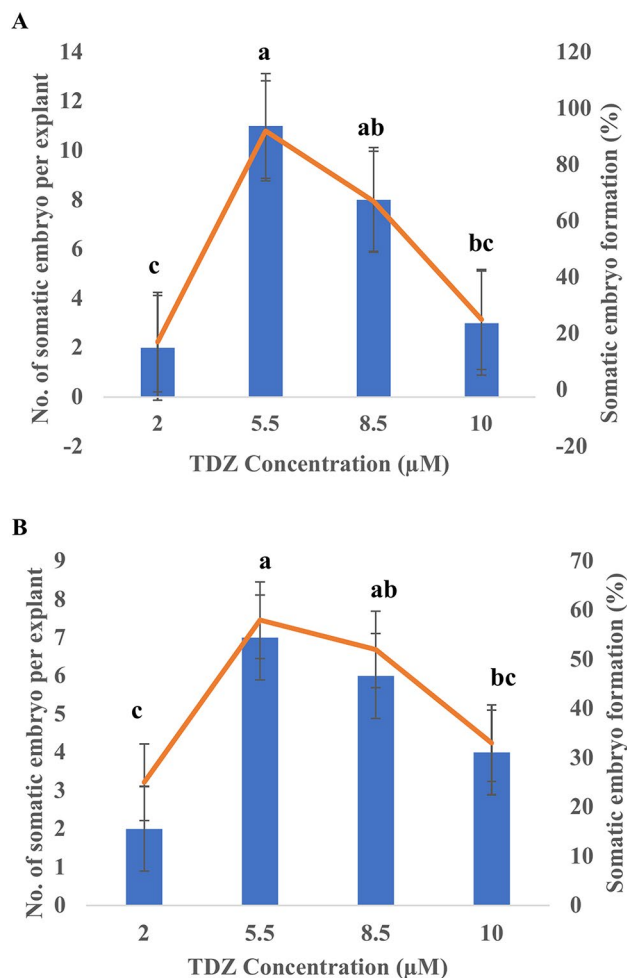


Fig. 2 Impact of TDZ concentrations (μM) on the number of somatic embryos per explant (represented by the primary axis in a column graph) and the percentage of embryo formation (represented by the secondary axis in a line graph) in both lingonberry cultivars after 9 weeks of culture. (A) and (B) show results for cultivars Cv1 and H1, respectively. Experiments were performed in triplicate, and significant differences were assessed using Tukey's test at $P \leq 0.05$. Different letters denote significant differences as determined by Tukey's test

medium containing 30 mL BM supplemented with $4.0 \mu\text{M}$ ZEA to promote plantlet maturation. After 12 weeks of culture, complete plantlets with both shoots and roots were observed (Fig. 3A). An additional week of growth led to well-developed shoots measuring 3–4 cm and roots measuring 1–2 cm (Fig. 3B). By this stage, plantlets reached lengths of 5–6 cm, featuring 7–11 leaves per shoot in the Cv1-5.5 (Fig. 3C). Similarly, growth in the H1-5.5 group was evident (Fig. 3D, E).

Healthy, vigorous plantlets were successfully regenerated in Sigma bottles labeled Cv1-5.5, Cv1-8.5, H1-5.5, and H1-8.5. In contrast, no significant growth or maturation was observed in plantlets from bottles labeled Cv1-2, Cv1-10, H1-2, and H1-10. Due to their reduced vigor, these plantlets were excluded from subsequent experiments. Thus, the

somatic embryogenic regenerant plants were Cv1-5.5, Cv1-8.5, H1-5.5 and H1-8.5. The data on the treatments applied, number of shoots per explant, shoot height (cm), number of leaves per shoot, shoot vigor, and root vigor were taken after 14 weeks of culture period (Table 2). Among the regenerated plants, Cv1-5.5 exhibited the highest shoot proliferation rate, with an average of 22.5 shoots per explant. It also has the highest shoot height (5.6 cm) and number of leaves per shoot (28.5). Statistical analysis revealed that both TDZ concentration and genotype had significant effects ($P \leq 0.05$) on shoot and root formation. Additionally, with one-way ANOVA statistically significant effect was observed with the treatment on number of shoots per explant ($p = 0.013$), shoot height ($p = 0.0004$), number of leaves per shoot ($p = 0.021$), shoot vigor ($p = 0.032$), and root vigor ($p = 0.015$) (Table 2).

Clonal fidelity assessment

Clonal fidelity assessment was done with young leaves from greenhouse maintained donor plants (Cv1-G, H1-G) and 12 week old leaves from SE regenerated lingonberry plants (Cv1-5.5, Cv1-8.5, H1-5.5, H1-8.5). 10 molecular markers including 5 EST-SSR and 5 GSSR were used. No polymorphic bands were detected in any of the examined samples. Altogether, 16 monomorphic DNA bands were present from 10 molecular markers. The number of bands generated by markers CA23, NA741, CA421, VCC_J9, and VCC_I2 was 1 band (1 amplified allele), whereas markers NA824, NA41, VCC_K4 and VCC_J1 generated 2 bands (2 amplified alleles) and VCC_S10 generated 3 bands (2 amplified alleles). A representative figure including 2 markers VCC_S10 (3 bands) and NA824 (2 bands) is shown in Fig. 4. GeneTools software from Syngene was used to analyse the number of bands obtained by using the 10 molecular markers.

Total phenolic content (TPC)

The various genetic compositions, methods of propagation, and their interactions had a significant impact (with a statistical significance of $P \leq 0.05$). The quantities of phenolic compounds per milligram, as well as the mean and variation (standard deviation) of these compounds in both donor plants and SE plants, are presented in Fig. 5A. The TPC levels in lingonberry plants followed a descending pattern: For the Cv1, the order was 'Cv1-G' ($0.39 \pm 0.02 \text{ mg GAE/g fw}$) $\geq$ 'SE'Cv1-5.5' ($0.37 \pm 0.04 \text{ mg GAE/g fw}$) $>$ 'SE'Cv1-8.5' ($0.21 \pm 0.00 \text{ mg GAE/g fw}$). Similarly, for the H1 type, the sequence was 'H1-G' ($0.41 \pm 0.00 \text{ mg GAE/g fw}$) $\geq$ 'SE'H1-5.5' ($0.41 \pm 0.00 \text{ mg GAE/g fw}$) $\geq$ 'SE'H1-8.5' ($0.37 \pm 0.00 \text{ mg GAE/g fw}$). Notably, 'Cv1-G' plants and 'SE'Cv1-5.5' exhibited higher TPC content compared to the SE plant

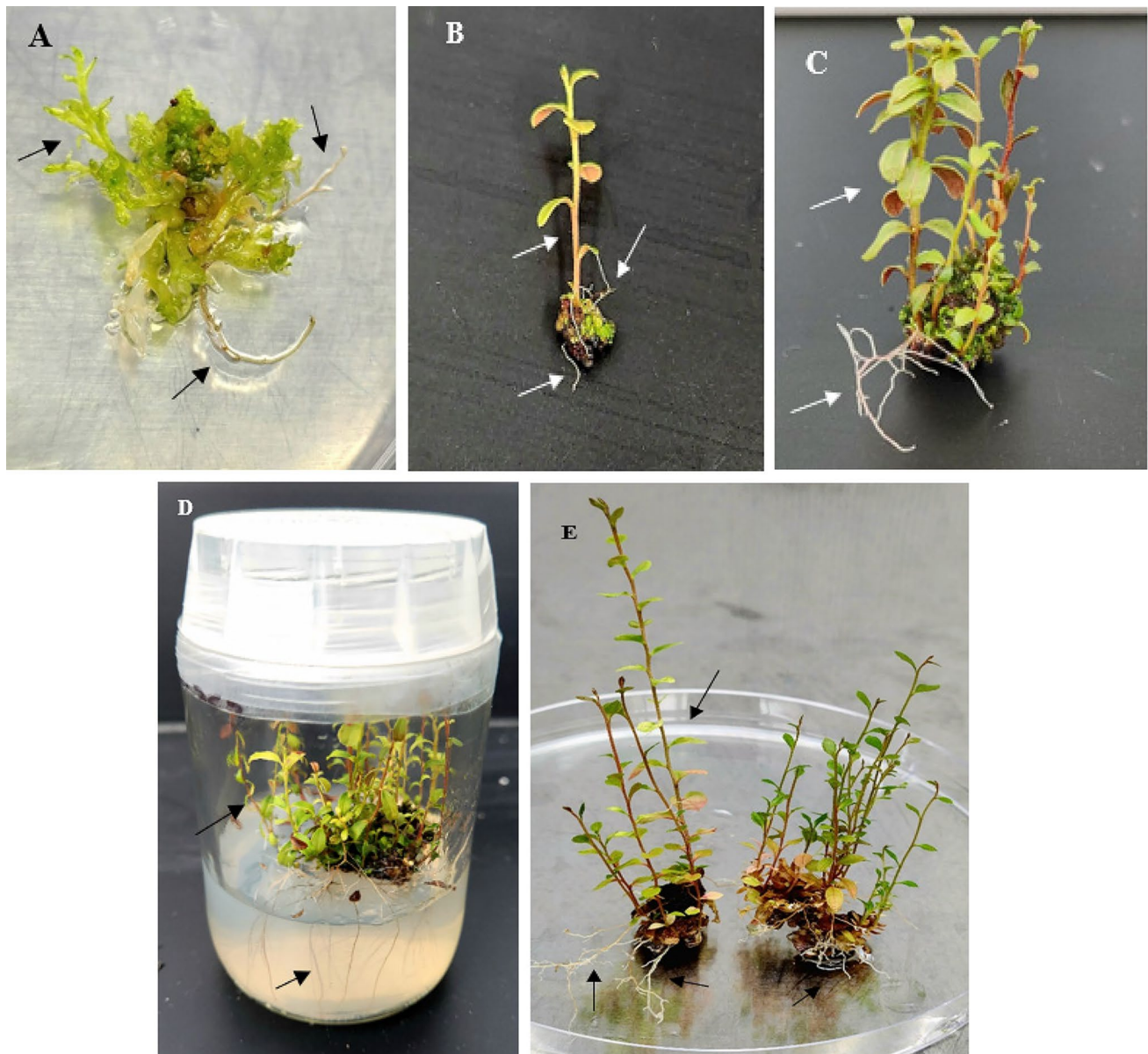


Fig. 3 (A) Germination and formation of complete plantlets with shoots and roots after 12 weeks of culture. (B) Well-developed shoots (3–4 cm) and roots (1–2 cm). (C) Plantlets (Cv1-5.5) reaching 5–6 cm

with 7–11 leaves per shoot. (D, E) Healthy growth of shoots and roots with leaves observed in somatic embryo regenerated H1-5.5 plantlets

SE'Cv18.5', although the TPC content in donor plants was slightly higher than that in SE-regenerated plants or almost the same. In the case of the H1 type, 'H1-G' and the SE plants demonstrated nearly identical TPC amounts (Fig. 5A).

Total flavonoid content (TFC)

The various genetic compositions, methods of propagation, and their interactions had a significant impact (with a statistical significance of $P \leq 0.05$). The quantities of flavonoid compounds per milligram, as well as the mean and variation (standard deviation) of these compounds in both

donor plants and SE plants, are presented in Fig. 5B. The TFC in lingonberry plants followed a descending pattern: For the Cv1 type, the order was SE'Cv1-5.5' (0.08 ± 0.00 mg CE/g fw) > 'Cv1-G' (0.06 ± 0.00 mg CE/g fw) > SE'Cv1-8.5' (0.04 ± 0.00 mg CE/g fw). Similarly, for the H1 type, the sequence was SE'H1-5.5' (0.09 ± 0.00 mg CE/g fw) > 'H1-G' (0.08 ± 0.00 mg CE/g fw) = SE'H1-8.5' (0.08 ± 0.00 mg CE/g f). Notably, SE'CV1-5.5' plants exhibited higher TFC compared to the donor plants. In the case of the H1 type, it also showed similar data, and the TFC of 'H1-G' and the SE plant 'H1-8.5' demonstrated identical amounts (Fig. 5B).

Table 2 Data on somatic embryo-regenerated lingonberry plants: number of shoots per explant, shoot height, number of leaves per shoot, shoot vigor, and root vigor in both the lingonberry genotypes grown in-vitro after a 14-week culture period

Treatment	Number of shoots per explant	Shoot height (cm)	Number of leaves per shoot	Shoot vigor	Root vigor
Cv1-5.5	22.5±1.2a	7.6±0.2a	21.5±1.5a	7.8±0.3a	6.6±0.2a
H1-5.5	21.0±1.0a	5.9±0.2b	20.7±1.3a	7.1±0.2b	5.1±0.2b
Cv1-8.5	18.3±0.8b	5.4±0.1c	17.7±1.0b	6.9±0.2b	5.6±0.2b
H1-8.5	17.0±0.7b	4.7±0.1c	15.9±0.9b	6.7±0.2b	5.1±0.2b

Values represent mean±standard error (SE) (n=3). Different letters within a column indicate significant differences among treatments according to Tukey's test at $P \leq 0.05$. Shoot vigor and root vigor was scored on a scale of 1 (necrotic and/or malformed shoots) to 8 (fully normal and healthy shoots with excellent vigor)

Total antioxidant activity (TAA)

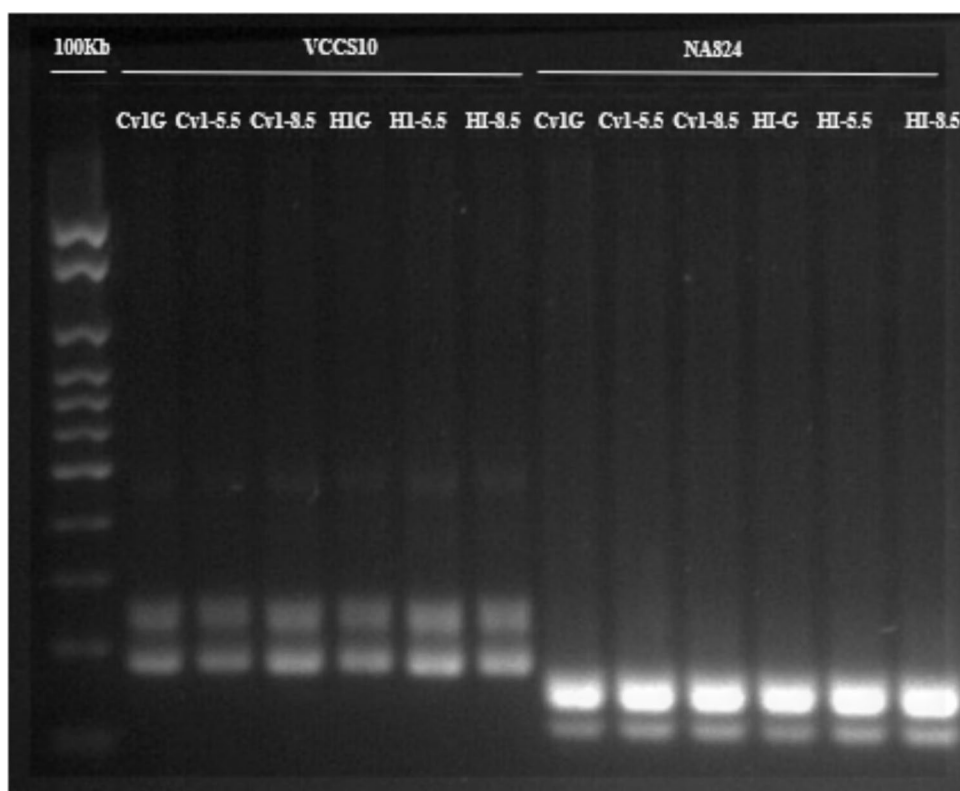
The diverse genetic compositions, propagation methods, and their interactions yielded a significant impact (with a statistical significance of $P \leq 0.05$). The levels of antioxidative activity per milligram, along with the mean and variance (standard deviation) of these substances in both donor and SE plants, are displayed in Fig. 5C. Antioxidant quantities in lingonberry plants followed a descending sequence: For the Cv1 type, the arrangement was SE'Cv1-5.5' (0.56 ± 0.12 mg GAE/g fw) $\geq$ SE'Cv1-8.5' (0.53 ± 0.04 mg GAE/g fw) $>$ 'Cv1-G' (0.51 ± 0.09 mg GAE/g fw). Correspondingly, for

the H1 type, the pattern was SE'H1-5.5' (0.81 ± 0.04 mg GAE/g fw) $\geq$ 'H1-G' (0.70 ± 0.05 mg GAE/g fw) and 'H1-G' (0.70 ± 0.05 mg GAE/g fw) $\geq$ SE'H1-8.5' (0.59 ± 0.03 mg GAE/g fw). Importantly, SE plants exhibited greater and almost similar TAA compared to the donor plants of both types. In the instance of the H1 type, SE'H1-5.5' demonstrated higher TAA than the SE plant 'H1-8.5' (Fig. 5C).

Anthocyanin assay (ACN)

The diverse genetic compositions, propagation methods, and their interactions yielded a significant impact (with a statistical significance of $P \leq 0.05$). The levels of ACN per milligram, along with the mean and variance (standard deviation) of these substances in both donor plants and SE plants, are displayed in Fig. 5D. ACN in lingonberry plants followed a descending sequence: For the Cv1 type, the arrangement was SE'Cv1-5.5' (0.73 ± 0.15 mg/g) $>$ SE'Cv1-8.5' (0.17 ± 0.19 mg/g) $\geq$ 'Cv1-G' (0.09 ± 0.1 mg/g). Correspondingly, for the H1 type, the pattern was SE'H1-5.5' (1.44 ± 0.13 mg/g) $>$ 'H1-8.5' (0.63 ± 0.05 mg/g) $\geq$ SE'H1-G' (0.42 ± 0.66 mg/g). Hence, SE plants for both types of lingonberries exhibited greater ACN compared to the donor plants (Fig. 5D).

Fig. 4 Agarose gel image showing DNA banding pattern in donor (Cv1-G) and somatic embryo (SE) regenerated plants in lingonberry cultivar. The first lane is the 100 bp DNA ladder. Monomorphic banding pattern was observed with markers VCC_S10 and NA824 with donor plant Cv1-G and H1-G and the somatic embryo regenerated plants Cv1-5.5, Cv1-8.5, H1-5.5 and H1-8.5



Antioxidant enzyme assay

The varying genetic compositions, methods of propagation, and their interactions had a notable influence (with statistical significance indicated by $P \leq 0.05$). The levels of antioxidant enzymes, CAT, SOD, and GR, as well as the average and range (standard deviation) of these substances, were assessed in both donor and SE plants. In terms of Cv1 genotypes, SE plants exhibited greater CAT and SOD activity compared to the donor plants, while for GR activity, donor plants demonstrated higher reductase activity than their SE counterparts (Fig. 6A, B, C).

Acclimatization success and growth performance of somatic embryo regenerated lingonberry plants under growth chamber condition

After 14 weeks of in-vitro culture period, the healthy somatic embryo regenerated plants were carefully taken out from the sigma bottles by removing excess agar medium and transplanted into black plastic pots ($25 \times 18 \times 6$ cm) (Fig. 7). The pots were filled with a mixture of 3 parts peat to 1 part perlite (v/v) at 20 ± 2 °C and 85% humidity (Barua et al. 2025) in growth chamber for maintenance and future experiments. The data on success and growth performance of somatic embryo regenerated lingonberry plants were recorded at regular monthly intervals. Table 3 presents the growth and survival data collected after 30 weeks of acclimatization.

Discussion

The study marks the first successful establishment of SE as a method for micropropagation in lingonberries (*Vaccinium vitis-idaea* L.). Lingonberry is a woody plant and regenerating it is quite challenging. The occurrence of embryogenesis followed by successful regeneration is quite uncommon, especially in *Vaccinium* species (berry crops) (Bhattacharya et al. 2010). This study represents a major breakthrough in the field of plant biotechnology, focusing on the lingonberry, a superfruit crop with both commercial and medical value. The SE protocol developed in this present study provides a scalable, effective, and genetically stable substitute for current propagation methods, addressing their drawbacks, including plant health, tissue lignification, genetic instability, and hyperhydricity. Lingonberry mostly has low rates of shoot proliferation and root formation during in vitro propagation due to its woody nature. Thus there is a significant challenge for propagating it compared to other berry crops like blueberry, cranberry, strawberry and other woody plants as well. SE proves to be advantageous in micropropagation as it allows cost-effective mass propagation and formation

of healthy shoots and roots. Two distinct genotypes, labeled as Cv1 and H1, were used for the initial phase of the study. Utilizing juvenile leaves as explants on a medium supplemented with 5.5 μ M TDZ, up to 92% embryogenic callus induction was achieved. Through scanning electron microscopy and histological investigations, the development and maturation of somatic embryos was verified, emphasizing the structural changes typical of SE. The protocol's resilience is demonstrated by the capacity to produce healthy plantlets from these embryos. The study advances the field by investigating the phytochemical profiles and clonal fidelity of the SE regenerated plants in addition to the novel SE approach in lingonberry. Compared to donor plants, SE-regenerated plants showed higher concentrations of flavonoids, anthocyanins, and antioxidant activity, making them better options for nutritional and therapeutic uses. This is consistent with the growing interest in lingonberries as a "superfruit" due to its potential health benefits. The use of TDZ concentrations was influenced by prior studies on woody plant species as well as the innate resistance of lingonberry in vitro regeneration (Bhat et al. 2024). Strong cytokinin-like growth regulator TDZ has been demonstrated to successfully stimulate somatic embryogenesis and shoot regeneration in a number of hard-to-regenerate species, such as half-high blueberry (*Vaccinium corymbosum* L. $\times$ *V. angustifolium* Ait) (Ghosh et al. 2018), apricot rootstock (*Prunus armeniaca* L.) (Elmoreigi et al. 2025) and *Atropa acuminata* (Bhat et al. 2024). To investigate a range that strikes a balance between morphogenetic efficacy and physiological stress, concentrations ranging from 2 to 10 μ M were chosen. As lingonberry is a recalcitrant woody plant, its propagation and regeneration is a significant challenge. TDZ has been used to induce somatic embryos from leaf explants due to its ability to mimic both auxin and cytokinin functions, making it highly effective on growth and differentiation of cultured explants (Govindaraj 2018). Its thiadiazolyl urea (Pai and Desai 2018) structure enhances cytokinin activity, promoting cell division, differentiation, and morphogenesis. TDZ influences the metabolism of plant growth regulators (PGRs) like auxins, ethylene, cytokinins and abscisic acid (ABA), affecting endogenous hormone levels which are crucial for somatic embryogenesis. Since the auxin-to-cytokinin ratio plays a vital role in callus formation, TDZ was added which provided the benefits of both auxin and cytokinin without the need for separate supplementation (Pai and Desai 2018). Thus, by adjusting or changing in endogenous hormone response during cell division, TDZ creates a favorable environment for callus formation and its transition into somatic embryos.

In this study four different TDZ concentrations were used, however 5.5 μ M TDZ turned to be the superior one. It was the optimal concentration where the hormone

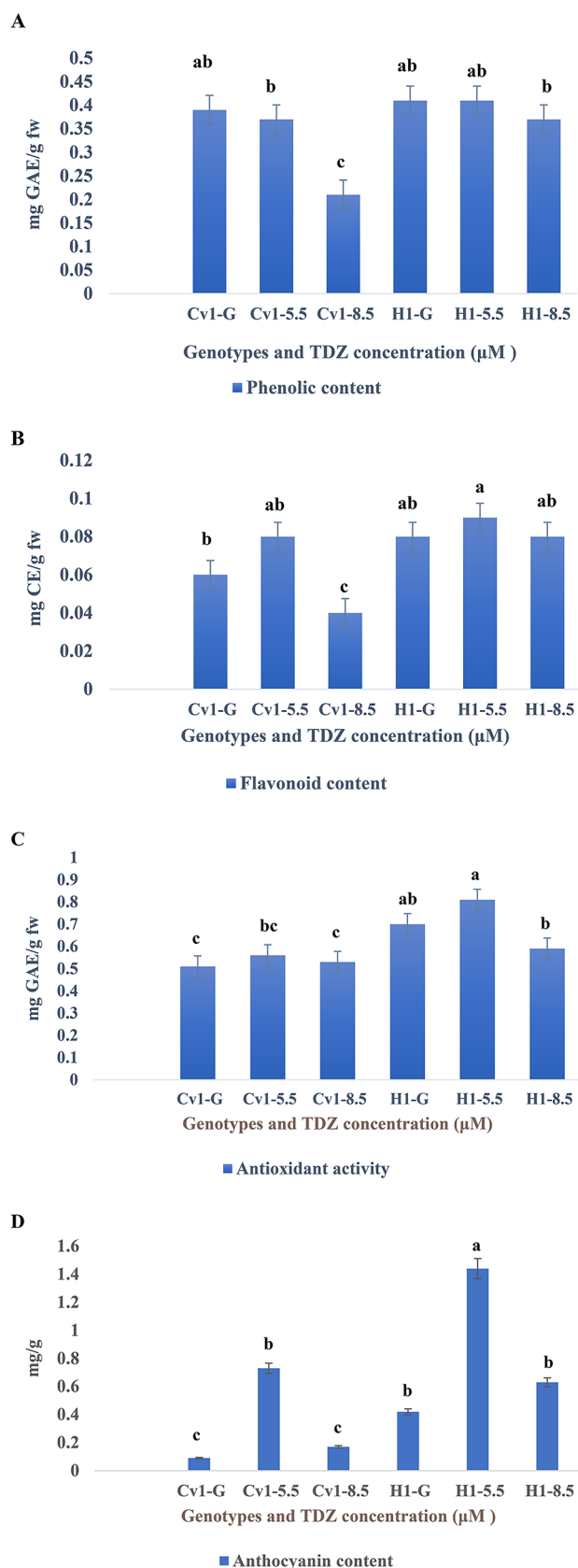


Fig. 5 Effects on cultivars and somatic embryo regenerated plants on phenolic, flavonoid, antioxidant, anthocyanin of both lingonberry genotypes. GAE=gallic acid equivalent, fw=fresh weight, CE=catechin equivalent, DPPH=2,2-diphenyl-1-picrylhydrazyl radical, G=donor plant, Donor plants (Cv1-G and H1-G), and somatic embryo regenerated plants developed from cultivar Erntedank and the hybrid plant using 4.0 μM zeatin (Cv1-5.5, Cv1-8.5, H1-5.5 and H1-8.5). Each experiment was repeated 3 times. Data is expressed as means $\pm$ SE. Values with the different letters in the same column are significantly different at $P \leq 0.05$ by Tukey's test

balance was ideal, leading to the highest number of somatic embryos. This concentration optimally stimulates cell division and differentiation, activating key genes and specific cellular pathways associated with embryogenesis. Additionally, TDZ may have triggered stress response pathways, enhancing the adaptability and resilience of cultured tissues, further supporting embryo induction.

Therefore, with a high success rate (92%) and a low risk of side effects such callus browning, low proliferating rates and low shoot–root vigor 5.5 μM TDZ provided the right hormonal conditions for efficient somatic embryogenesis in lingonberry, making it the most effective concentration for regeneration. Additionally, elevated TDZ concentrations can elicit stress responses, resulting in the accumulation of phenolic compounds and tissue necrosis. However, further increasing the concentration to 10 μM resulted in a decrease in embryo formation and poor vigor in shoots, roots, and leaves. Therefore, 5.5 μM TDZ was investigated to be the optimal concentration for producing well-developed somatic embryos, leading to the regeneration of high-fidelity lingonberry plant. While several hypotheses exist regarding the mechanism of action of TDZ, its precise mode of operation remains uncertain (Radhakrishnan et al. 2009). An effective procedure that was adapted to the particular difficulties presented by the woody character of lingonberries was developed by this approach. There were also prominent differences ($P \leq 0.05$) between the genotypes, where 'Cv1-5.5' had the highest percentage (92%) and 'H1-5.5' (83%), 'H1-8.5' (75%), 'Cv1-8.5' (67%) and others. Across genotypes the maximum SE formation was observed at 5.5 μM TDZ. ANOVA results revealed that TDZ concentrations significantly influenced the count of somatic embryos formed ($P \leq 0.05$) on the examined media. Specifically, at 2 and 10 μM TDZ, the number of embryos per explant ranged from 2 to 7, while at 5.5 and 8.5 μM TDZ, it varied from 21 to 17 in both genotypes. For Cv1, tukey's test showed that somatic embryo formation at 5.5 μM TDZ was statistically significant compared to 2 μM ($p=0.007$) and 10 μM ($p=0.019$). Similarly, a significant difference was observed between 2 μM and 8.5 μM TDZ ($p=0.041$). Similarly for H1, tukey's test showed that somatic embryo formation at 5.5 μM TDZ was significantly higher than at 2 μM TDZ ($p=0.038$). A similar significant increase was observed at

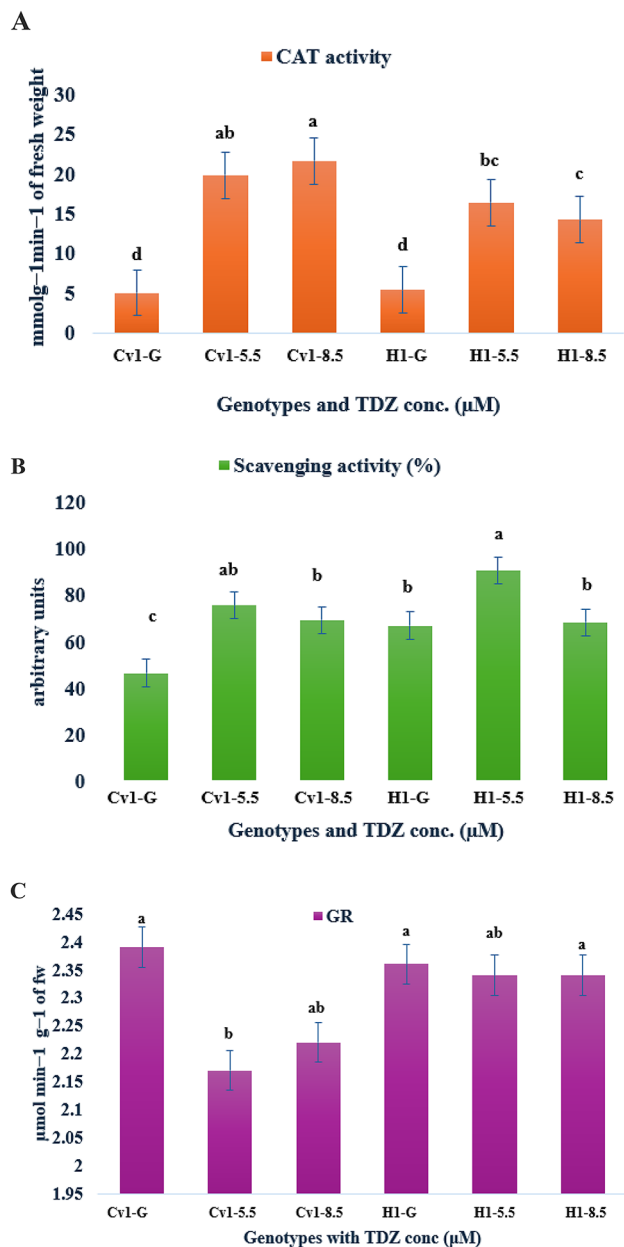


Fig. 6 Bar graphs with standard error of the antioxidant enzymes, (fw=fresh weight) **(A)** catalase activity, **(B)** superoxide dismutase (was determined as a scavenging activity (%)) in the presence of nitroblue tetrazolium), **(C)** glutathione reductase for all the lingonberry genotypes including donor plants (Cv1-G and H1-G), somatic embryo regenerated plants developed from cultivar Erntedank and the hybrid plant using 4.0 μM zeatin (Cv1-5.5, Cv1-8.5, H1-5.5 and H1-8.5). Each experiment was repeated 3 times. The significance of the difference is calculated with a one-way ANOVA analysis–Tukey test ($P \leq 0.05$). Standard errors associated with different letters indicate significant differences

8.5 μM TDZ compared to 2 μM TDZ ($p = 0.048$). Previously studies on in vitro propagation techniques for lingonberry included axillary shoot proliferation (Debnath 2005), shoot tip culture (Debnath and McRae 2001), shoot organogenesis

from hypocotyl segments (Debnath 2003), and shoot development from nodal explants (Debnath and McRae 2001). These traditional methods have been extensively applied to lingonberry micropropagation which served as a valuable reference for evaluating the efficacy of this newly applied SE protocol in lingonberry. The results obtained from SE enabled a comprehensive assessment of the relative efficiency and advantages of SE over the previously studied methods. The findings from the present study (Table 2) when compared to the earlier reports highlights the clear potential of somatic embryogenesis as a superior propagation strategy for recalcitrant lingonberries. This is because SE has its ability to regenerate both roots and shoots from a single embryogenic structure, enabling a rapid and mass regeneration process (Vahdati et al. 2004). Additionally, SE is a rapid vegetative propagation technique (Moradi et al. 2017), enabling the generation of multiple embryos from a single somatic explant, resulting in a higher number of plantlets produced in a shorter time frame compared to other methods like axillary shoot proliferation, which typically yield fewer shoots from existing buds. Such efficiency with the combination of producing true-to-type plantlets, reduces the risk of somaclonal variation which are often associated with organogenesis-based techniques (Sadat-Hosseini et al. 2019). The ability to generate large numbers of embryos from a single explant also enhances the speed and scalability of breeding programs.

The success and growth performance of somatic embryo regenerated lingonberry plants were monitored in growth chamber on regular monthly basis. Table 3 summarizes the survival rates and growth data recorded after 30 weeks of acclimatization (Fig. 7). The manuscript also highlights that plants regenerated via somatic embryogenesis were successfully acclimatized, with survival rates and growth performance assessed under controlled growth chamber conditions. The data on 30 weeks after transferring the in-vitro regenerated plantlets to ex-vitro conditions, provides valuable insights into the practical feasibility and effectiveness of the regeneration protocol that was implemented. Among the evaluated genotypes of lingonberry, Cv1 exhibited the highest survival rate of $92.5 \pm 3.0\%$ under 5.5 μM TDZ followed by H1-5.5, Cv1-8.5 and H1-8.5. Together with its plant height, leaf number, and total leaf area, Cv1's higher survival rate at 5.5 μM TDZ highlights its adaptability in vitro, making it a viable choice for future propagation and further research.

For successful commercial scaling of SE-regenerated plants, maintaining true-to-type regeneration is utmost essential. In this study, molecular markers (EST-SSR and GSSR) were used to assess the genomic integrity of regenerated plants. The results showed genetic stability, with monomorphic banding patterns in agarose gel, confirming

Fig. 7 Somatic embryo regenerated lingonberry plants: **(A)** Cv1 and **(B)** H1 after 30 weeks of acclimatization in growth chamber conditions

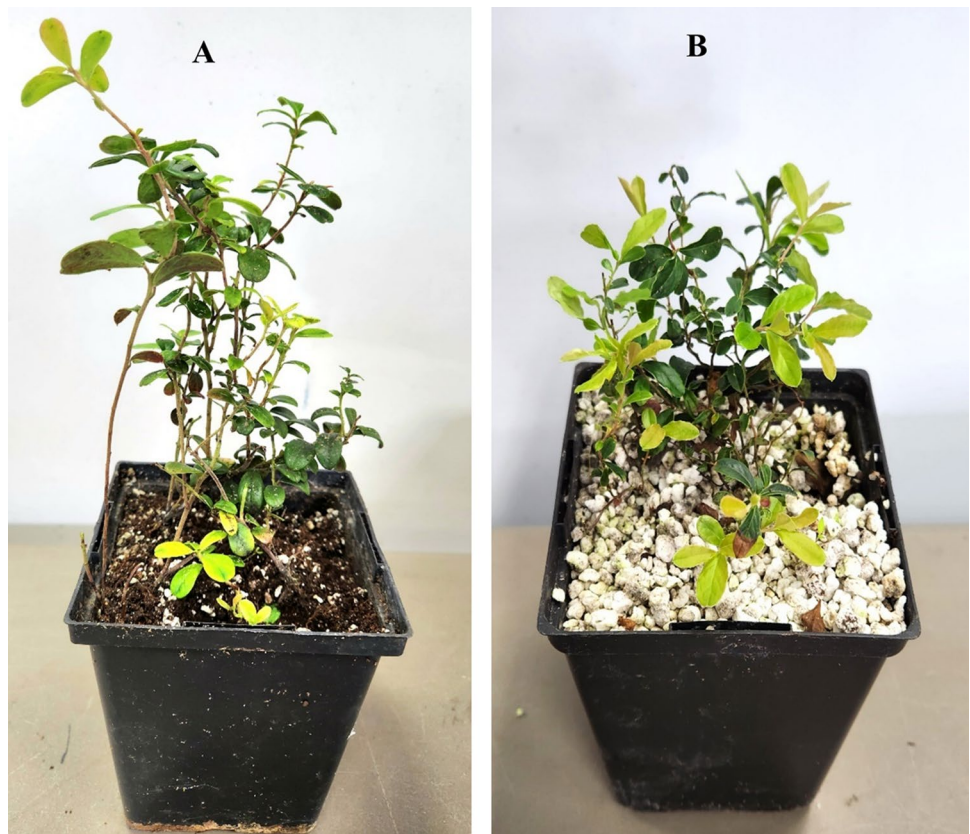


Table 3 The growth and survival data of somatic embryo regenerated lingonberry plants (Genotypes Cv1 and H1) after 30 weeks of acclimatization in growth chamber conditions

Parameter	Genotypes	5.5 μ M TDZ	8.5 μ M TDZ
Average plant height (cm)	Cv1	29.5 $\pm$ 1.2a	28.0 $\pm$ 1.0b
	H1	30.0 $\pm$ 1.1a	28.5 $\pm$ 0.9b
Leaf number	Cv1	6.5 $\pm$ 0.5a	5.8 $\pm$ 0.4b
	H1	7.0 $\pm$ 0.6a	6.2 $\pm$ 0.5b
Total leaf area (cm ²)	Cv1	45.6 $\pm$ 3.5a	38.2 $\pm$ 3.0b
	H1	42.8 $\pm$ 3.2a	36.5 $\pm$ 2.8b
Survival rate (%)	Cv1	92.5 $\pm$ 3.0a	82.5 $\pm$ 2.5b
	H1	90.0 $\pm$ 2.8a	80.0 $\pm$ 2.3b

(Mean $\pm$ SD). Different letters (a, b) used to indicate significant differences of two genotypes ($p < 0.05$) between treatments

clonal fidelity between donor and regenerated plants. Similar studies by Sadat-Hosseini et al. (2019) highlighted the significance of genetic stability in somatic embryo-regenerated plants to preserve desirable traits during micropropagation. These molecular markers are reliable tools to detect any somaclonal variation, confirming the effectiveness of this method in ensuring clonal fidelity for future breeding and conservation purposes.

Vaccinium berries are primarily renowned for their antioxidant activity, and for having enormous phenolic content, flavonoid content and anthocyanin content (Ghosh et al. 2018; Sikdar et al. 2022), which have a notable impact not

only on the plant's defense mechanisms but also on human health advantages. The most abundant group of phenolic plant chemicals, known as polyphenolic compounds, can be found throughout the leaves, fruits, seeds, and blossoms. These compounds vary in structure, ranging from uncomplicated components with a solitary hydroxylated aromatic ring to intricate polymeric substances (Debnath & Goyali 2020). In this research, we conducted a comparison of changes in TPC, TFC, TAA and ACN content between donor plants and plants regenerated through SE. According to the thorough phytochemical profiling conducted for the study, SE-regenerated plants outperformed donor plants in terms of anthocyanin levels, total flavonoid content, and antioxidant activity. These improvements raise the plants' nutritional and therapeutic worthwhile also raising the possibility of greater adaptability and stress tolerance. It's interesting to note that donor plants had a higher overall phenolic content, suggesting that the in vitro growing circumstances had an impact on several metabolic pathways. In the current study, TDZ stimulated the production of total flavonoids, anthocyanins, and antioxidant activity in the regenerated plants compared to the control plants, indicating its potential role in boosting secondary metabolite biosynthesis. This observation contrasts with findings by (Baskaran et al. 2012) which found that TDZ stimulates *Merwillia plumbea* total flavonoid content. Previous studies by Mok M et al. (2000) suggested

that TDZ can regulate the biosynthesis and metabolism of endogenous cytokinins. Thus it can influence the nutrient transport mechanisms within the plant. Such regulatory mechanisms are frequently associated with pathways linked to stress, suggesting that TDZ may initiate metabolic reprogramming due to stress. Jariteh et al. (2011) reported that stress conditions during in vitro culture enhance the induction of somatic embryogenesis in recalcitrant Persian walnut tissues. Similarly, in the present study, TDZ also acted as a oxidative stress mediator in recalcitrant lingonberry, triggering somatic embryo induction. This stress-mediated developmental reprogramming not only induced somatic embryo formation but also activated stress-responsive regulatory pathways, leading to enhanced secondary metabolite production in the regenerated plants.

On the other hand the main challenges faced in today's world is to continued production of enough food with high yield, disease resistance, and improved variety. However, a plant's natural environment is composed of a intricate set of abiotic stresses and biotic stresses. Plant responses to these stresses are equally intricate. Global climate changes can lead to significant declines in crop yield and quality (Kusvuran et al. 2016). Different plant species exhibit various physiological and biochemical reactions, as well as distinct levels of tolerance, when exposed to environmental stressors such as salinity, drought, extreme temperatures, herbicide exposure, and mineral deficiencies. This exposure disrupts the equilibrium between the generation of reactive oxygen species (ROS) and the counterbalancing action of antioxidants, often resulting in oxidative harm. Due to the potential disruption of normal metabolism caused by reactive oxygen species like superoxide ($O_2^{\cdot-}$), H_2O_2 , and the highly reactive hydroxyl radical ($\cdot OH$), which can cause oxidative damage to lipids, proteins, and nucleic acids, plants have evolved various antioxidant enzymes to shield against these harmful effects. Various enzymes in plant tissues scavenge ROS, such as the mitochondrial and chloroplast-localized enzymes SOD and GR, which regulate ROS levels. CAT an enzymatic antioxidant and it can remove or neutralizes or obliterates oxidative intermediates and free radicals (Debnath et al. 2013). The combined actions of SOD and CAT (Jaleel et al. 2009) play a role in preventing or reducing the formation of ROS (Kusvuran et al. 2016). Furthermore, two categories of nonenzymatic antioxidants exist: lipid-soluble, membrane-associated antioxidants (such as α -tocopherol and β -carotene) (Kusvuran et al. 2016), and water-soluble reductants (like glutathione and phenolics). GR enzymes (Scandalios 1993), crucial components of the defense mechanism in tolerant genotypes against salt, drought, and chilling stress, mainly operate in chloroplasts and mitochondria, effectively reducing H_2O_2 to water and thereby detoxifying these harmful substances. This research revealed a notable

increase in the concentrations of CAT and SOD in plants regenerated through SE compared to donor plants. Given the pivotal function of the cooperative effects of SOD and CAT in safeguarding plants against challenges such as defense mechanisms, aging, and oxidative stress, this observation holds significance (Jaleel et al. 2009). Despite the valuable functions of GR, its levels were slightly diminished in SE-regenerated plants. Furthermore, the possible combination of this SE process with genetic transformation methods may open the door to specific trait improvements, tackling issues like climate adaptation and disease resistance. Thus the current study optimized a method using thidiazuron (TDZ) and zeatin to produce genetically stable lingonberry plants with enhanced biochemical profiles, addressing the low propagation rate of woody plants. Comparative analysis between regenerated and donor plants revealed phytochemical stability, aligning with Sikdar et al. (2022), who showed that micropropagated lingonberry retains similar phytochemical profile both in-vitro and ex-vitro conditions through epigenetic regulation. Similar trends were reported by Debnath and Ghosh (2022); Fraga et al. (2016); Khan et al. (2021), highlighting that epigenetic memory and stress adaptation play key roles in preserving secondary metabolites. Future work incorporating transcriptomic analyses specially focusing on gene expression and epigenetic changes would provide deeper insights into developmental changes during SE and to further understand metabolic stability under ex vitro conditions.

Conclusion

This study reports the first application of somatic embryogenesis for in vitro propagation of lingonberries. Demand for lingonberries outpaces what can be harvested from the wild, owing in part to their documented health benefits, including for cardiovascular, renal and hepatic health. This is a huge opportunity to produce more. This demand calls for the rapid mass production of medicinal plant species, where somatic embryogenesis appears as a fast in-vitro micropropagation protocol and an essential technique needed for large scale propagation of target tissues. This presents a significant opportunity for increased production. Effective mass production is essential to meet the rising demand, and somatic embryogenesis emerges as a highly efficient in-vitro micropropagation technique for large-scale cultivation of lingonberry. TDZ was shown to enhance somatic embryogenesis in lingonberries, with the optimal concentration for producing the highest quality embryos at 5.5 μM , suggesting that using optimised concentration of TDZ during the early stages of somatic embryogenesis significantly enhances the efficiency of embryonic development. These

enhanced genetic fidelity and phytochemical profiles offer the added advantage of dual benefits, and this methodology emerges as a significant advancement in sustainable agriculture and plant biotechnology.

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Author contributions This is an Agriculture and Agri-Food Canada (AAFC) St. John's Research and Development Centre contribution. S.K. performed all experiments and drafted the manuscript. While S.C.D. conceived the idea, designed the study, and coordinated and supervised the experiments and analyses, and edited the revised manuscript; R.B. assisted in biochemical assay. H.D.M., A.U.I. and Y.L.S. participated in study design and contributed to the editing and revision of the manuscript. All authors read and approved the final manuscript.

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Data Availability All data generated or analyzed in this study are involved in this manuscript.

Declarations

Competing interests The authors declared that they have no competing interests.

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