



From disorder to recovery: unraveling hyperhydricity insights in *Vaccinium vitis-idaea* L. through phytochemical and gene expression studies

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Abstract

Vaccinium vitis-idaea L. (lingonberry) is a health promoting northern berry crop valued for its antioxidant properties and is commonly propagated through *in vitro* techniques. However, the success of micropropagation is often hindered by hyperhydricity (HH), a physiological disorder that causes shoots to become water soaked and malformed. This study is the first investigation of HH in the lingonberry cultivar ‘Erntedank’ (Cv1), using a combination of structural, biochemical, and gene expression analyses. Lingonberry shoots grown in both liquid and semi-solid culture media developed typical HH symptoms, including excessive water accumulation and distorted tissues. Treatment with potassium silicate (K_2SiO_3) significantly reduced water content and improved shoot appearance over time. Scanning electron microscopy (SEM) showed distorted stomata, trichomes, and vascular tissues in hyperhydric shoots, which were restored upon silicon treatment. Biochemical profiling, using ultra-high-performance liquid chromatography coupled with mass spectrometry (UHPLC-MS/MS), showed that HH increased the levels of stress related compounds, such as anthocyanins and phenolic acids, which were reduced after silicon treatment. Gene expression analysis using reverse transcription quantitative polymerase chain reaction (RT-qPCR) revealed an over 30-fold upregulation of antioxidant genes (CAT1, APX, and SOD) and ethylene related genes (ACO1, ACS11, COX2, and ETR1) in HH tissues, which were further downregulated following silicon treatment. Therefore, the findings from this study suggested that hyperhydricity in lingonberry is linked to oxidative and hormonal stress, and that silicon supplementation offers a promising strategy to prevent or recover from this disorder, improving the efficiency and quality of lingonberry micropropagation.

Keywords Lingonberry micropropagated plant · Hyperhydricity · Scanning electron microscopy · Potassium silicate · UHPLC-MS/MS · RT-qPCR

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Introduction

Vaccinium vitis-idaea L. ($2n=24$), commonly known as lingonberry, is a circumboreal, evergreen shrub valued for its edible berries, medicinal properties, and ornamental appeal (Vander Kloet 1988). Widely distributed across Northern and Central Europe, North America, and parts of Asia, it includes two major subspecies, *vitis-idaea* and *minus*, with variations in growth habits and fruiting behavior (Gustavsson 2001; Debnath and Arigundam 2020). The berries are rich in phenolic compounds, flavonoids, triterpenoids, and essential fatty acids, offering antioxidant, anti-inflammatory, cardioprotective, and neuroprotective benefits (Isaak *et al.* 2017; Kowalska 2021; Vilkickyte *et al.* 2020). Given the rising demand for this nutraceutical crop, micropropagation provides a valuable platform for large scale propagation, conservation of elite genotypes, and yr-round production under controlled conditions.

One of the key limitations in the micropropagation process is hyperhydricity (HH), a physiological disorder characterized by excessive water uptake, brittle tissues, translucent leaves, and reduced shoot vigor. HH is commonly induced by factors such as high relative humidity, hormonal imbalances, or limited gas exchange within sealed culture vessels (Ziv 1991; Hazarika 2006). It severely affects shoots morphology and function by impairing stomatal development, reducing lignin content, thinning the cuticle, and damaging chloroplasts and mitochondria (Ziv 1991; Picoli *et al.* 2001; Marques *et al.* 2021). In micropropagated plants, HH impairs gas exchange and photosynthesis, leading to poor growth and survival (Polivanova and Bedarev 2022). Reactive oxygen species (ROS), such as hydrogen peroxide and hydroxyl radicals, accumulate in hyperhydric tissues, aggravating oxidative damage (Jaspers and Kangasjärvi 2010). Hyperhydricity conditions have been observed in more than 200 plant species, including *Vaccinium* species, such as blueberry (Gao *et al.* 2018; Barua *et al.* 2025), and significantly hampers large scale propagation and acclimatization efforts (Hazarika 2006; Ziv 1991).

Recent advances highlight the potential of silicon (Si) as a non-toxic, stress alleviating agent in plant tissue culture. Though not considered essential, silicon is beneficial under stress, contributing to cell wall strengthening, improved water relations, enhanced organogenesis, and mitigation of ROS accumulation (Sivanesan and Park 2014). Silicon supplementation using potassium silicate (K_2SiO_3) has been shown to reverse HH in crops like *Dianthus caryophyllus* L. by modulating stress responsive gene expression and restoring structural integrity (Soundararajan *et al.* 2017).

To address the lack of targeted studies, this research presents the first comprehensive investigation of hyperhydricity in micropropagated lingonberry, integrating morphological,

anatomical, biochemical, and molecular approaches. Scanning electron microscopy (SEM) was used to assess ultrastructural alterations in leaves and stems between hyperhydric and recovered tissues. To evaluate biochemical disruptions, ultra-high-performance liquid chromatography coupled with mass spectrometry (UHPLC-MS/MS) was used, which is a high-resolution analytical technique (Mustafa *et al.* 2022), to quantify key phytochemicals, including anthocyanins (for example cyanidin-3-glucoside), flavonols (quercetin, rutin), flavan-3-ols ([+]-catechin, [−]-epicatechin), procyanidins (B1 and B2), and phenolic acids (chlorogenic, gallic, vanillic). These compounds are essential for antioxidant defense and contribute to the nutritional and therapeutic value of lingonberry.

At the molecular level, to analyze gene expression patterns, reverse transcription quantitative polymerase chain reaction (RT-qPCR) was used, a sensitive technique for measuring transcript levels of stress associated genes. The study focused on 10 genes implicated in oxidative stress response, ethylene biosynthesis, and cell wall metabolism. To better understand the molecular basis of hyperhydricity in lingonberry, the expression of catalase isozyme 1 (CAT1), superoxide dismutase (SOD) (Muneer *et al.* 2017), ascorbate peroxidase (APX), and cytochrome c oxidase subunit 2 (COX2), which are crucial for maintaining redox homeostasis and mitochondrial function under stress, were investigated (Gao *et al.* 2017; Mansilla *et al.* 2018). Alcohol dehydrogenase (ADH12) is associated with anaerobic metabolism and cellular hypoxia, conditions frequently linked to gas accumulation in sealed culture vessels (Rojas-Martínez *et al.* 2010). The ethylene biosynthesis pathway, often activated in hyperhydric shoots, involves 1-aminocyclopropane-1-carboxylic acid synthase (ACS1) and oxidase (ACO1), both of which can promote tissue vitrification by affecting cell division and water retention (Sreelekshmi *et al.* 2022). Additionally, peroxidase 12 (PER12) contributes to metabolic regulation, (Ribeiro *et al.* 2019), while ethylene receptor 1 (ETR1) regulates ethylene perception and downstream stress responses (Ziv 1991; Kevers *et al.* 2004). GDP-mannose pyrophosphorylase (GMP) is essential for cell wall precursor biosynthesis (Mounet-Gilbert *et al.* 2016) structural features significantly altered in hyperhydric shoots. Therefore, by combining structural, biochemical, and molecular analyses, this study provides new insights into the mechanism driving hyperhydricity and offers a practical strategy for improving the efficiency of lingonberry micropropagation systems.

Materials and methods

Plant material and culture conditions The lingonberry genotype used in this study, the cultivar ‘Erntedank’ (Cv1) (Kundu *et al.* 2025), was grown and maintained at the St. John’s Research and Development Centre (SJRDC),

Agriculture and Agri-Food Canada, St. John's, Newfoundland and Labrador, Canada. Formal plant identification was done by Dr. Samir C. Debnath. Young, actively growing shoots (3 to 4 wk old) were harvested from greenhouse maintained donor plants (Cv1), grown in black plastic pots ($10.5 \times 10.5 \times 12.5$ cm) filled with a 3:1 (v/v) peat-to-perlite mix. Greenhouse conditions included natural light (maximum $90.0 \mu\text{mol m}^{-2} \text{s}^{-1}$), 20 ± 2 °C temperature, and 85.0% relative humidity (Debnath 2017). Stem segments (4 to 6 cm) were rinsed under running tap water, followed by a 2 min wash in 2.0% (v/v) mild liquid detergent. Surface sterilization was carried out in 1.05% sodium hypochlorite solution (prepared from 20.0% commercial bleach) with 0.1% Tween for 25 min, then briefly rinsed with 70.0% ethanol, and washed three times with sterile deionized water. Sterilized segments were trimmed into three-node sections and cultured aseptically on a modified Murashige and Skoog (MS; Murashige and Skoog 1962) medium containing three-quarter micro salts and macro salts and vitamins of Debnath and McRae's (2001) basal medium supplemented with (per liter) 3.5 g Sigma A 1296 agar, 25.0 g sucrose, and 1.25 g Gelrite™ (Sigma Chemical Co., St. Louis, MO). For shoot induction, semi-solid and liquid media were used. Both the media were further enriched with zeatin ($4.5 \mu\text{M}$). The pH was adjusted to 5.0. Semi-solid medium (35.0 mL) was dispensed into 175.0 mL Sigma baby food jars, following Ghosh *et al.* (2018). For liquid culture, 200.0 mL of medium was used in stationary bioreactors (Growtek™, 1.178 L; 15 cm height $\times$ 5.7 cm diameter; Fisher Scientific, Ottawa, Canada) (Barua *et al.* 2025). The Growtek bioreactor features a rotating, floating explant holder with a supportive matrix, a side tube for medium exchange, and a durable polycarbonate body. Five explants were placed per vessel, with subculturing performed every eight wk in both liquid and semi-solid media. In semi-solid medium, 3.5 g L⁻¹ Sigma A 1296 agar (Sigma Chemical Co.) and 1.25 g L⁻¹ Gelrite (Sigma Chemical Co.) were added. Both the media were sterilized by autoclaving at 121 °C for 15 min. All culture vessels were sealed with Parafilm. They were incubated at 24 ± 2 °C under a 16 h photoperiod with a photosynthetic photon flux density of $30.0 \mu\text{mol m}^{-2} \text{s}^{-1}$ provided by cool white, fluorescent lamps.

Silicon treatment Previous studies have demonstrated the effectiveness of silicon (Si) in mitigating hyperhydricity in plant tissue cultures (Sivanesan *et al.* 2011). Building on this evidence, hyperhydric lingonberry shoots (Fig. 2A, B) were cultured for four wk on MS medium containing macro- and micro-nutrients, following the formulation of Debnath and McRae (2001). The medium was supplemented with 25.0 g L⁻¹ sucrose and solidified using 0.8% agarose. In the current study, potassium silicate (K₂SiO₃) (Thermo Scientific, Mississauga, Canada) was used as the silicon source

at a concentration of 1.2 mM, which was determined to be the most effective dose based on preliminary trials. The pH of the medium was adjusted to 5.0 prior to autoclaving at 121 °C for 15 min.

Water content To measure the water content of the normal and hyperhydric lingonberry shoots, fresh leaf samples were selected and weighed, and their fresh weight (FW) was recorded. Then, they were dried at 70 °C in oven, and their dry weights (DWs) were recorded at intervals over 180 min. Water content of the samples was calculated using the following formula (Barua *et al.* 2025):

$$\text{Water content (\%)} = 100\% \times [(FW - DW)/FW]$$

Scanning electron microscopy (SEM) To visualize the stomata, trichome, and organization of vascular bundle scanning electron microscope (SEM) (JEOL JSM7100F field emission SEM equipped with JEOL SE/BSE detectors, Deben CL detector and Thermo EDS system) (JEOL, ON, Canada), the study was conducted on 6-wk-old proliferated *in vitro* shoots and 30-wk-old *ex vitro* plant samples. Both leaf and stem samples were cut into 1 cm² and 1.0 cm pieces and subsequently fixed overnight in a 0.1 M phosphate buffer (pH 7.2) containing 2.5% glutaraldehyde (Thermo Scientific). The samples were dehydrated in graded series of ethanol and then were dried in a desiccator for 12 h. Finally, the samples were gold coated before the micrograph observation.

Phytochemical analysis - Reagents and standards Anthocyanins (cyanidin- β -glucoside), procyanidin B1, procyanidin B2, flavonols (quercetin, rutin, myricetin), flavan-3-ols ((+)-catechin, (-)-epicatechin), and phenolic acids (chlorogenic acid, gallic acid, vanillic acid, p-coumaric acid, ferulic acid and methyl gallate), thus all the standards, were purchased from Millipore-Sigma, Oakville, Canada. Individual stock solutions of each analyte, at a concentration of 1000.0 mg L⁻¹, were freshly prepared by dissolving pure standards in HPLC-grade methanol (Fisher Scientific). Formic acid (99.0%) and analytical-grade hydrochloric acid (37.0%) were obtained from Fisher Scientific. Deionized water was used. All solvents and solutions were filtered through a 0.22 μm filter (Millex®). Before HPLC analysis, all samples were filtered with Millex® 33 mm 0.22 μm filter.

Sample preparation and extraction for UHPLC-MS/MS measurements For extraction of the biochemical compounds, 2.0 g of leaf from each sample were extracted with 10.0 mL of ethanol:water mixture (70:30, v/v) acidified with HCl (1.5%) for 60 min at 25 °C. Followed by centrifugation at 5000 rpm for 10 min with a ThermoFisher Scientific Centrifuge (Thermo Scientific) (ST 8R), the sample solutions were filtered through a 0.22 μm filter before injection into

HPLC–MS/MS triple quadrupole. All samples were stored at -15°C until analysis. Each sample was analysed in triplicates.

Gene expression analysis - RNA extraction and cDNA synthesis Total RNA extraction was performed using a Qia-gen Plant mini kit (Qiagen, Hilden, Germany). Approximately 100.0 mg of plant material was used, to which 1.0% β -mercaptoethanol was added, and the extraction was done following the manufacturer's instructions. NanoDrop® (JENWAY; Genova Nano, Fisher Scientific) spectrophotometry was used to assess the integrity of the isolated RNA; in addition, samples were analyzed on a 1.0% agarose gel (1X TAE buffer), pre-stained with GelRed (Sigma-Aldrich) (0.5 mg mL^{-1}). After total RNA purification, DNase treatment was carried out to eliminate potential genomic DNA contamination using the Turbo™ DNase kit (Applied Biosystems, Mississauga, ON, Canada). Also, cDNA was synthesized using the iTaq universal SYBER Green One-Step kit (BIO-RAD, Mississauga, Canada) and was used for RT-qPCR.

Gene selection and primer design A total of 10 genes potentially involved in hyperhydricity were selected from the literature, including LOC105037251(SOD) (Muneer *et al.* 2017), ETR1, (Gao *et al.* 2022), PER12 (Ribeiro *et al.*

2019), ACS11, ACO11, APX, COX2, ADH 12, CAT1 (Lelis *et al.* 2025), and GMP, as well as the reference gene actin (Table 1) After gene selection, gene sequence accessions were used to search for lingonberry orthologous genes in NCBI. The Primer3plus software was used to design the primers using the parameters of average temperature range of 57 to 63°C for each primer. Absence of amplification and nonspecific products were evaluated using the Oligo-Analyzer 3.1 software. RT-qPCR with $\Delta\Delta\text{Ct}$ analysis was done to measure relative gene expression of the selected hyperhydric genes.

Gene expression study by RT-qPCR Relative gene expression analysis was performed for 8 genes potentially involved in hyperhydricity on a StepOnePlus 96-well Real-Time PCR Systems (Applied Biosystems). All reactions were comprised of $10.0\text{ }\mu\text{L}$ iTaq universal SYBR® Green reaction mix (BIO-RAD), $0.2\text{ }\mu\text{L}$ of each primer at an initial concentration of 10.0 mM , and $2.0\text{ }\mu\text{L}$ of single stranded cDNA for each sample to be analyzed (diluted $20\times$).

Data analysis Data are expressed as mean $\pm$ standard deviation from three replicates. Statistical analyses were conducted using IBM SPSS Statistics version 29.0.2.0. Differences among treatment means were assessed using Tukey's HSD test, with a significance threshold set at $p \leq 0.05$.

Table 1. Gene primers for hyperhydricity analysis in RT-qPCR

Gene	GenBank Accession	Primers (5' to 3')	TM ($^{\circ}\text{C}$)
<i>ETR1</i>	XM_010923296.3	F: AATGGGCTTGATACGGACAC R: AGTGCACATTCTCCAAACC	59.8/60
<i>CAT1</i>	XM_010919942.3	F: CCCGTTTGGAACAACAACCTC R: CCTCTTGCATGAACAACACG	60.4/60.3
<i>ADH12</i>	XM_068465421.1	F: GCGAGGAGGGATGATTACAG R: TAGTCCTAGAAGCAGCAGCG	60.2/60
<i>SOD (LOC105037251)</i>	XM_010912937.2	F: GCTTCATGGCTTCCATGTTC R: AGTCGCATTCCCAAGATCAC	60.6/60.1
<i>PER12</i>	XM_010912689.3	F: ATAGCCTCACCTTCGCCTCT R: GGTGGTGTGCGAGGTTGATCT	60.4/60
<i>COX2</i>	YP_009472098.2	F:GGGTCTTGGTTCGCGCTTTA R:GCTGGATCGACTACTACCTCGT	60/60
<i>APX</i>	NM_001281059.1	F:GCATTCCGATCACTCTCTGC R: ACGGAGCATAATTGGAGCAC	59.8/60
<i>ACS11</i>	NM_116873.2	F:TTCAAATGGGTCTCGCCGAA R: GTAACCTGTGTGGACGGGGT	60/60
<i>ACO1</i>	NM_001247095.2	F:CAGCAGCTTGTGTGCAATCC R: TCGGACCGGCTTTTGGTTTA	60/60
<i>GMP</i>	NM_001247096.3	F:GCCCCCTTGTGATTTTGGTA R: CGGTCTCTTGAGAGCATCTG	60/60
<i>Actin*</i>	CL5740.Contig2_All	F:ACCTTTTGGATTGTGGGCTAGA R: AGCTCCACTTGCACCTTTTCCTT	60/60

ETR1- ethylene receptor 1, *CAT1*- catalase isozyme 1, *ADH12*- alcohol dehydrogenase, *SOD*- superoxide dismutase, *PER12*- peroxidase 12, *COX2*- cytochrome c oxidase subunit 2, *APX*- ascorbate peroxidase, *ACS11*-1-aminocyclopropane-1-carboxylic acid synthase, *ACO1*-1-aminocyclopropane-1-carboxylic acid oxidase, *GMP*- GDP mannose pyrophosphorylase

Statistical significance was determined at a 95.0% confidence level ($\alpha=0.05$). Raw data from all runs of RT-qPCR were used to perform $\Delta\Delta C_t$ analysis to determine relative gene expression of the selected hyperhydric genes. The normalization was performed using internal reference genes (Actin). The plots and heatmaps were generated using R studio (R.4.4.2 version).

Results

Water content The analysis of water content across treatments revealed significant differences ($p \leq 0.05$), indicating the impact of silicon supplementation on the reduction of hyperhydricity. Hyperhydric Growtek-grown shoots had the highest water content (Cv1-HHG, 86.0%), while hyperhydric *in vitro* sigma-grown shoots had the second highest water content (Cv1-HHS, 77.0%). After 4 wk, the potassium silicon treated shoots on Growtek (liquid medium) Cv1-SiG1 had a water content of 75.0%, while on Sigma (semi-solid medium) Cv1-SiS1 was 70.3%. Both displayed a moderate decrease in water content following silicon treatment. Growtek (Cv1-SiG2) and Sigma (Cv1-SiS2) had a water content of 63.0% and 61.6%, respectively, after 8 wk, which were statistically distinct from all other treatments, highlighting their effectiveness in mitigating hyperhydricity. These results confirmed that silicon, particularly at higher concentrations, significantly reduced excess water accumulation in lingonberry shoots, thus alleviating hyperhydric symptoms (Fig. 1).

Morphology and hyperhydricity recovery in micropropagated lingonberry shoots

Hyperhydricity is a significant

challenge during micropropagation of lingonberries, with visibly altered plant morphology observed under both liquid (Growtek) and semi-solid (Sigma) media. As seen in Fig. 2A and B, affected shoots exhibited classic hyperhydric symptoms like thickened, blackish, and brittle stems, short internodes, and translucent, curled, and fragile leaves. In severe cases, shoots became twisted, blackened, and necrotic, with a stunted, glassy appearance, and began drying out from the third wk of culture, especially in liquid conditions. In order to mitigate the problem, 1.2 mM K_2SiO_3 was introduced into the medium after 3 wk. While necrotic tissues did not revive, partially affected shoots began showing signs of improvement after 4 wk of silicon treatment (Fig. 2C, D). Gradually, new green shoots emerged from the surviving bases, with a marked improvement in leaf architecture and turgor in both media types. By the 16th wk, a clear and sustained recovery of hyperhydric shoots were observed in both the media (Fig. 2E, F). The silicon treated shoots developed healthy, non-glassy leaves, elongated internodes, and a vigorously growing shoot system, visually distinct from their earlier hyperhydric state. Among all tested concentrations, 1.2 mM K_2SiO_3 emerged as the most effective for reversing hyperhydricity, supporting steady shoot regeneration and physiological balance. This recovery trajectory from initial damage to visible renewal over 16 wk underscored the potential of silicon as a targeted, non-toxic intervention to mitigate culture induced disorders in micropropagated lingonberry.

Scanning electron microscopy (SEM) SEM provided detailed insight into the cellular level differences between hyperhydric, and silicon treated micropropagated lingonberry shoots. It revealed distinct anatomical changes associated with physiological disorder and the subsequent recovery.

Figure 1. Water content hyperhydric shoots of lingonberry (*Vaccinium vitis-idaea* L.) genotype Cv1, in liquid (Growtek; Cv1-HHG), and semi-solid (Sigma; Cv1-HHS) and after 4 wk and 8 wk of silicon treated non-hyperhydric shoots in liquid (Growtek; Cv1-SiG1, Cv1-SiG2 respectively), and semi-solid (Sigma; Cv1-SiS1; Cv1-SiS2 respectively). Data represents the mean $\pm$ standard deviation (SDs). Experiments were performed in triplicate, and significant differences were assessed using Tukey's HSD test at $P \leq 0.05$. Different letters denote significant differences as determined by Tukey's HSD test.

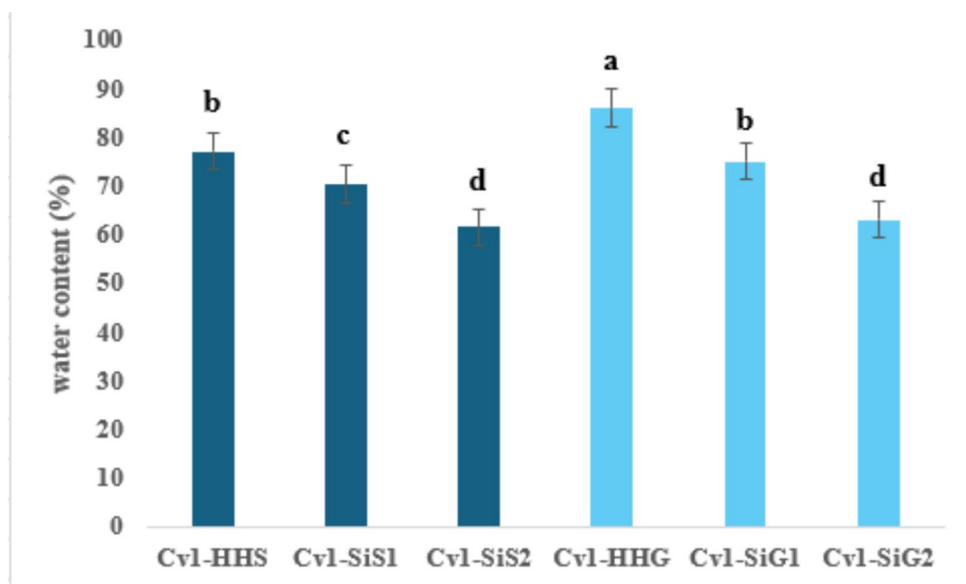
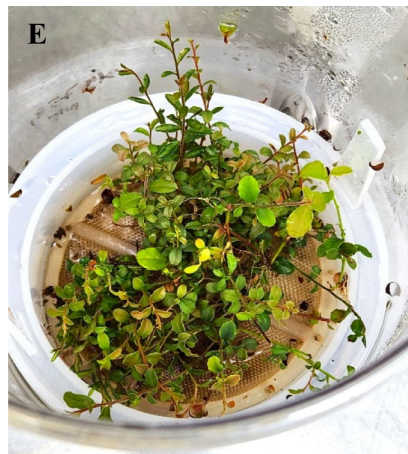
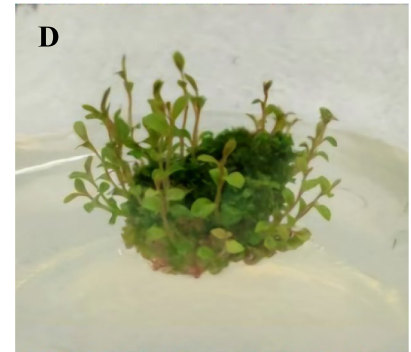
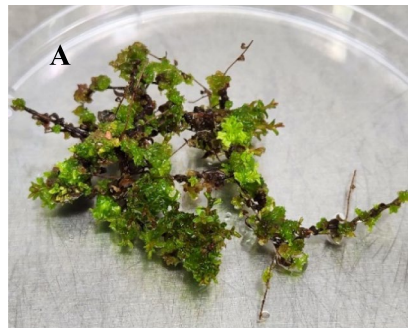


Figure 2. Hyperhydric (control) and potassium silicate treated non-hyperhydric shoots of micropropagated lingonberry (*Vaccinium vitis-idaea* L.) (Cv1). (A) 3-wk-old hyperhydric shoots grown in Growtek (liquid) medium showed darkened, brittle stems and poorly developed shoots with blackish discoloration. (B) in Sigma jar (semi-solid) medium, shoots displayed short internodes with translucent, curled, and fragile leaves. (C) after four wk of potassium silicate treatment, non-hyperhydric shoots in liquid medium began developing new shoots with tiny emerging leaves (D) similarly early recovery were observed in semi-solid medium. (E) healthy, non-glassy leaves, elongated internodes, and a vigorously growing shoot system in liquid and (F) semi-solid medium after 16 wk.



Leaf surface and epidermal architecture In hyperhydric shoots cultured in Growtek (liquid) and Sigma (semi-solid) media, SEM analysis of the leaf surface revealed severe anatomical distortions. Epidermal cells were disorganized, with irregular surface textures, loss of cell definition, and distended shapes, consistent with excessive water retention. The stomata were thickened and disoriented in shapes (Fig. 3A, C). Nonglandular trichomes, which play a protective role in normal tissues, were notably fewer in number, deformed, or curled into coiled structures (Fig. 3E, G), making them functionally compromised. These trichomes lacked the structural integrity required for proper defense and transpiration regulation.

By contrast, silicon treated hyperhydric shoots after four wk of 1.2 mM potassium silicate supplementation displayed remarkable anatomical restoration. Newly formed leaves exhibited well-organized epidermal cells, a smoother, well-defined surface, and healthy, erect nonglandular trichomes with normal morphology (Fig. 3F, H). Stomatal development was also notably improved (Fig. 3B, D), with distinct, elliptical stomata and well-formed guard cells, suggesting better regulation of gas exchange. These improvements were consistently observed in both media, highlighting silicon's role in restoring cellular structure and surface morphology.

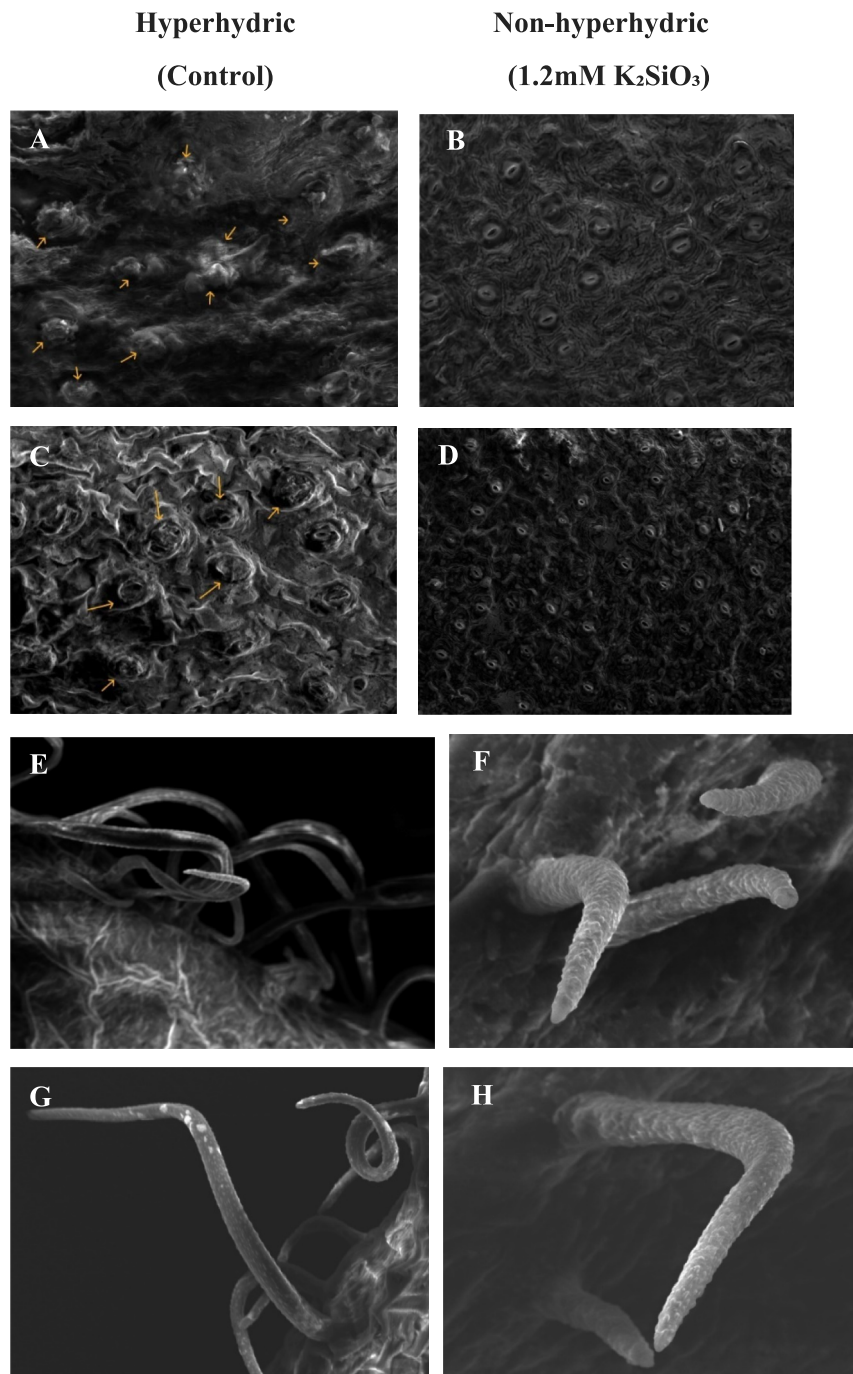


Figure 3. Scanning electron microscopy of lingonberry (*Vaccinium vitis-idaea* L.) genotype Cv1 showing hyperhydric (control) and silicon treated (non-hyperhydric) tissues. (A) mesophyll tissue with disoriented and thickened stomata in leaf cultured on Growtek medium (liquid) after 3 wk. (B) organized mesophyll tissue and healthy stomata in silicon treated micropropagated shoots on liquid medium after 4 wk. (C) mesophyll tissue with disoriented and thickened stomata in leaf cultured on Sigma jar (semi-solid) medium after 3 wk. (D) organized mesophyll tissue and healthy stomata in silicon treated shoots on semi-solid medium after 4 wk. (E) coiled and disoriented trichomes in liquid medium. (F) well-developed and organized trichomes in silicon treated shoots in liquid medium. (G) coiled and disoriented trichomes in semi-solid medium. (H) well-developed and

organized trichomes in silicon treated shoots in semi-solid medium. (I) stem surface of hyperhydric shoots in liquid medium. (J) stem surface of silicon treated healthy shoots in liquid medium. (K) stem surface of hyperhydric shoots in semi-solid medium. (L) stem surface of silicon treated healthy shoots in semi-solid medium. (M) stem cross-section showing irregular xylem and phloem with large intercellular spaces under hyperhydric conditions in liquid medium. (N) normal xylem and phloem without enlarged intercellular spaces in silicon treated shoots in liquid medium. (O) stem cross-section showing irregular xylem and phloem with large intercellular spaces under hyperhydric conditions in semi-solid medium. (P) normal xylem and phloem in silicon treated shoots in semi-solid medium.

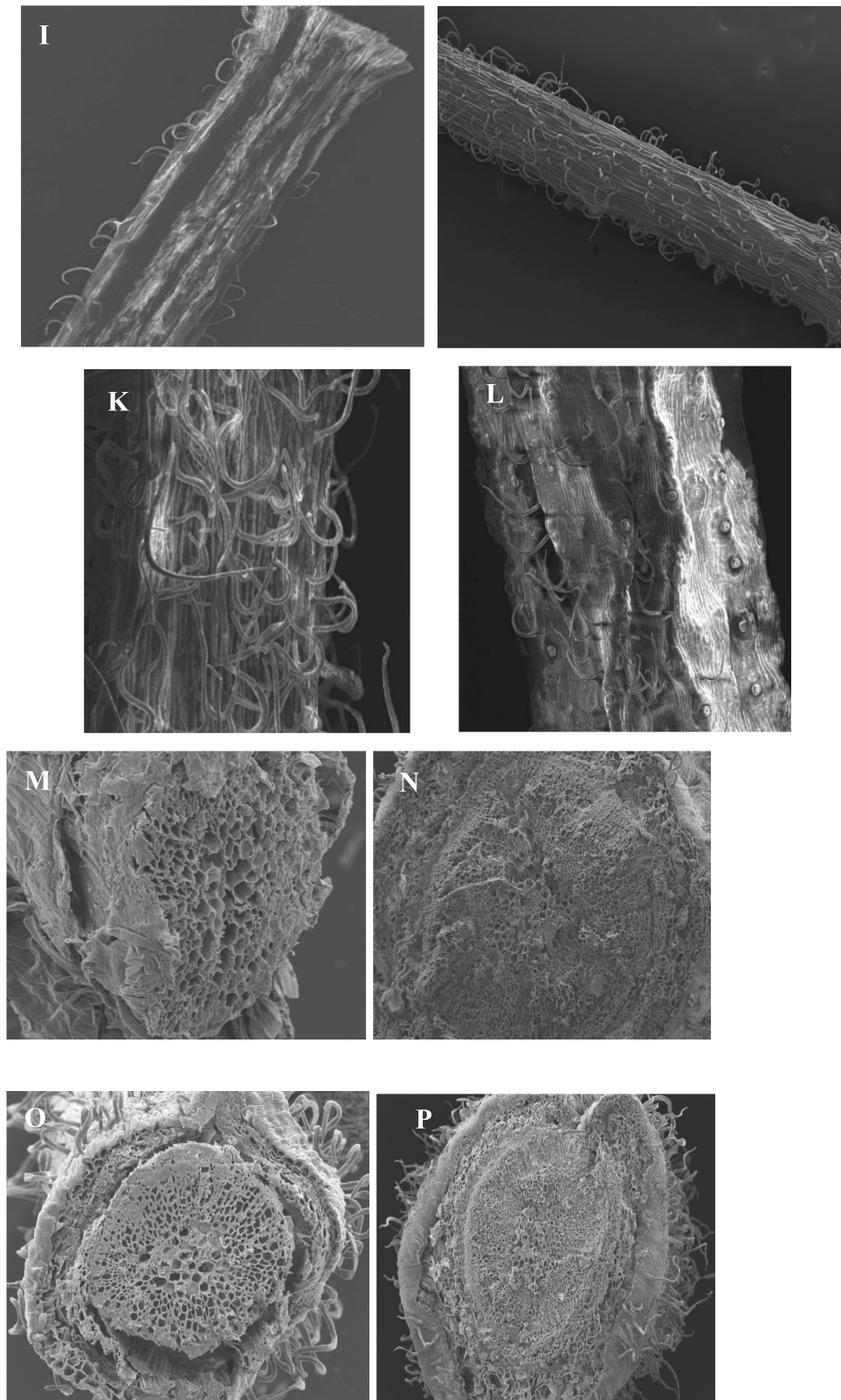


Figure 3. (continued)

Stem anatomy and vascular bundle organization Similar patterns were observed in stem cross-sections. In hyperhydric tissues, stems showed disoriented and malformed trichomes, with reduced density and coiled shapes, mirroring leaf abnormalities (Fig. 3I, K). More critically, SEM images revealed disrupted vascular organization: irregularly arranged xylem and phloem (Fig. 3M, O), thin and fragile cell walls, and excessively enlarged intercellular spaces (Fig. 3M, O). These structural deficiencies contributed to the spongy texture of hyperhydric stems and likely impaired water and nutrient transport, reinforcing the visible stunted growth and necrosis seen macroscopically. After silicon treatment, stems exhibited restored trichomes, and surface orientation (Fig. 3J, L), vascular integrity, with well-organized vascular bundles, compact xylem and phloem alignment, and thicker, structurally sound cell walls (Fig. 3N, P). The intercellular spaces were reduced, and trichomes on the stem surface regained their elongated, non-coiled form, suggesting improved tissue rigidity and water conducting efficiency.

Optimization of UHPLC UHPLC-MS analysis was optimized for the detection and quantification of flavonoids, anthocyanins, and phenolic acids using a binary gradient system, coupled with a Triple Quadrupole Mass Spectrometer (TQ-MS), and operated in Dynamic Multiple Reaction Monitoring (dMRM) mode. Chromatographic separation was achieved using a reverse phase column maintained at 30 °C, with a total run time of 12.5 min. A binary solvent system was used comprising water with 0.1% formic acid (Solvent A) and methanol or acetonitrile with 0.1% formic acid (Solvent B), delivered at a constant flow rate of 0.3 mL min⁻¹. The elution gradient started at 80% A and 20% B, transitioned to 15% A and 85% B at 7.3 min, and returned to initial conditions at 8 min, optimizing compound separation and reducing peak broadening and retention time shifts. The injection mode was set to injection with needle wash using a 3 µL injection volume, with a draw speed of 100 µL min⁻¹, ejection speed of 400 µL min⁻¹, and wash time of

3 s. These conditions ensured minimal sample carryover and high reproducibility. The TQ-MS system utilized Agilent Jet Stream Electrospray Ionization (AJS-ESI) in both positive and negative modes. Source parameters were finely tuned for each polarity, and compound specific transitions, fragmentor voltages, and collision energies were optimized to ensure sensitive and selective quantification. A calibration curve with multi-level external calibration standards across twelve concentration levels, ranging from 5 ng mL⁻¹ to 10,000 ng mL⁻¹ was used. Regular maintenance including solvent quality checks and system suitability testing ensured method robustness throughout the study.

Phytochemical profiling of hyperhydric and non-hyperhydric lingonberry shoots UHPLC-MS/MS analysis was conducted to assess the impact of hyperhydricity and silicon based recovery on secondary metabolite accumulation in lingonberry. Ten key phytochemicals, classified into anthocyanins, flavan-3-ols, flavonols, phenolic acids, and procyanidins, were quantified. Statistical analysis using ANOVA followed by Tukey's HSD test revealed significant differences across treatment groups ($p \leq 0.05$), with detailed comparisons presented below (Table 2 and Fig. 4). UHPLC-MS/MS chromatograms are presented in supplementary Figure S1.

Anthocyanin content The concentration of cyanidin-3-glucoside was significantly elevated in hyperhydric tissues, peaking at 21.83 ± 0.13 mg kg⁻¹ FW in Growtek (Cv1-HHG) and 19.46 ± 0.20 in Sigma (Cv1-HHS) hyperhydric shoots. Silicon treatment markedly reduced these levels to 11.66 ± 9.49 (Cv1-SiG) and 10.70 ± 0.03 (Cv1-SiS). All differences were statistically significant ($p \leq 0.05$).

Procyanidins Both procyanidin B1 and B2 exhibited strong and significant upregulation in hyperhydric conditions. Procyanidin B1 reached 9.31 ± 8.06 and 8.67 ± 8.09 in Growtek and Sigma hyperhydric shoots, respectively, and was reduced to 6.18 ± 8.36 and 2.53 ± 2.44 following K₂SiO₃ treatment. Procyanidin B2 showed the highest accumulation

Table 2. Comparison of hyperhydric and non-hyperhydric (Silicon treated) lingonberry (*Vaccinium vitis-idaea* L.) plants in both culture media (Growtek: liquid and Sigma: semi-solid)

No	Compound	Hyperhydric (Growtek)	Hyperhydric (Sigma)	Si treated (Growtek)	Si treated (Sigma)
1	Cyanidin-3-glucoside	21.83 ± 0.13	19.46 ± 0.20	11.66 ± 9.49	10.70 ± 0.03
2	Procyanidin B1	9.310 ± 8.06	8.676 ± 8.09	6.188 ± 8.36	2.532 ± 2.44
3	Procyanidin B2	169.93 ± 27.9	137.08 ± 22.6	97.24 ± 15.7	92.73 ± 13.8
4	Quercetin	18.22 ± 1.41	15.63 ± 1.22	10.53 ± 0.80	9.99 ± 0.63
5	Rutin	14.55 ± 1.34	4.07 ± 0.41	5.33 ± 0.62	3.33 ± 0.19
6	(+)-Catechin	546.86 ± 65.3	544.16 ± 49.3	183.62 ± 7.8	190.62 ± 9.3
7	(-)-Epicatechin	358.69 ± 32.4	203.38 ± 15.9	198.20 ± 14.6	171.27 ± 13.3
8	Chlorogenic acid	767.91 ± 63.2	522.05 ± 63.0	256.10 ± 20.6	204.72 ± 19.5
9	Vanillic acid	2.867 ± 2.44	2.131 ± 1.77	1.427 ± 1.25	1.253 ± 1.05
10	Gallic acid	0.381 ± 0.03	0.370 ± 0.02	0.356 ± 0.03	0.353 ± 0.02

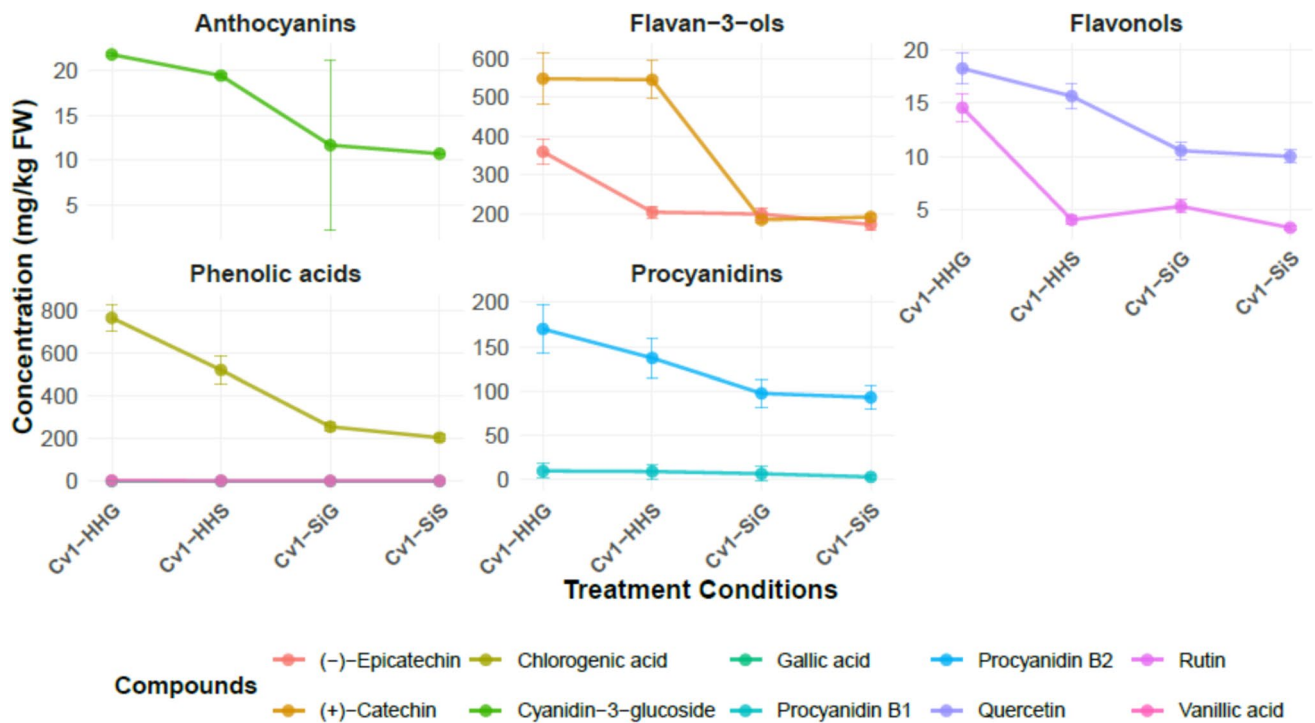


Figure 4. Phytochemical profiles of micropropagated lingonberry (*Vaccinium vitis-idaea* L.) shoots under different hyperhydric and silicon treatment conditions. The bar plots represent the concentration (mg kg^{-1} FW) of key secondary metabolites categorized as anthocyanins (cyanidin-3-glucoside), flavan-3-ols ((+)-catechin and (-)-epicatechin), flavonols (rutin and quercetin), phenolic acids (chlorogenic acid, gallic acid, vanillic acid), and procyanidins (B1 and B2). Treat-

ments include hyperhydric plants grown in Growtek (liquid) medium (Cv1-HHG), hyperhydric plants in Sigma jar (semi-solid) medium (Cv1-HHS), silicon treated plants in Growtek (Cv1-SiG), and Sigma (Cv1-SiS) medium. Results are expressed as mean $\pm$ standard deviation of three biological replicates. Significant reductions in most compounds were observed after silicon treatment compared to hyperhydric tissues (ANOVA, Tukey's HSD, $p \leq 0.05$).

among procyanidins, with 169.93 ± 27.90 (Cv1-HHG) and 137.08 ± 22.60 (Cv1-HHS), dropping significantly upon Si application to 97.24 ± 15.70 (Cv1-SiG) and 92.73 ± 13.80 (Cv1-SiS), indicating partial biochemical normalization.

Flavonols Among flavonols, quercetin content increased significantly in hyperhydric samples (18.22 ± 1.41 in Cv1-HHG and 15.63 ± 1.22 in Cv1-HHS) and was reduced after K_2SiO_3 treatment to 10.53 ± 0.80 and 9.99 ± 0.63 in Growtek and Sigma, respectively. Rutin displayed a similar pattern, with hyperhydric levels at 14.55 ± 1.34 in Cv1-HHG, which significantly declined in the K_2SiO_3 -treated group (5.33 ± 0.62) and showed a more dramatic drop in Cv1-HHS (4.07 ± 0.41) and its corresponding Si treatment (3.33 ± 0.19). The decrease was statistically significant ($p \leq 0.05$), though more variable in Sigma conditions.

Flavan-3-ols (+)-Catechin exhibited one of the highest responses, increasing more than threefold in hyperhydric conditions (546.86 ± 65.30 in Cv1-HHG and 544.16 ± 49.30 in Cv1-HHS) compared to its reduced values under

K_2SiO_3 -treatment (183.62 ± 7.8 and 190.62 ± 9.30 , respectively). Similarly, (-)-epicatechin was highly elevated in Cv1-HHG (358.69 ± 32.40) and Cv1-HHS (203.38 ± 15.90) and significantly decreased with K_2SiO_3 -treatment (198.20 ± 14.60 and 171.27 ± 13.30).

Phenolic acids Chlorogenic acid was the most abundant phenolic compound detected, with concentrations as high as 767.91 ± 63.20 in Cv1-HHG and 522.05 ± 63.00 in Cv1-HHS. K_2SiO_3 -treated shoots showed a substantial decline to 256.10 ± 20.60 (Cv1-SiG) and 204.72 ± 19.5 (Cv1-SiS). Vanillic acid also followed this trend, increasing in hyperhydric tissues (2.86 ± 2.44 in Cv1-HHG and 2.13 ± 1.77 in Cv1-HHS) and decreasing with K_2SiO_3 -treatment (1.42 ± 1.25 and 1.25 ± 1.05), though with larger standard deviations. In contrast, gallic acid remained stable across all treatments, ranging narrowly from 0.35 ± 0.02 to $0.38 \pm 0.03 \text{ mg kg}^{-1}$ FW, and did not show significant differences ($p > 0.05$). Overall, 9 out of the 10 tested compounds showed statistically significant increases in hyperhydric conditions ($p \leq 0.05$), with partial but significant biochemical

restoration observed in silicon treated groups. Gallic acid was the only compound that did not exhibit statistically significant variation across treatments. The results suggested a strong link between hyperhydricity and elevated accumulation of antioxidant related phytochemicals, particularly chlorogenic acid, catechins, procyanidins, and flavonols, and indicated the potential of silicon supplementation to modulate these biochemical responses.

Gene expression analysis of hyperhydricity related genes using RT-qPCR In the present study, RT-qPCR was performed to investigate the expression of 10 genes associated with oxidative stress, ethylene signaling, and metabolism in normal and hyperhydric lingonberry shoots. The analysis used the $2^{-\Delta\Delta Ct}$ method with Actin as the reference gene. Statistical significance was determined using ANOVA followed by Tukey's HSD ($p \leq 0.05$). The gene expression heatmap of 10 genes in both media are shown in Fig. 5.

Eight genes showed statistically significant upregulation in hyperhydric shoots (Cv1-HHG) relative to silicon-treated shoots (Cv1-SiG) when cultured in liquid medium. Among the antioxidant related genes, CAT1 showed the highest expression increase with a 35.0-fold upregulation ($\Delta\Delta Ct = -5.13$, $\log_2 FC = 5.13$, $p = 0.002$), followed closely by APX at 32.0-fold ($\Delta\Delta Ct = -5.00$, $\log_2 FC = 5.00$, $p = 0.001$), SOD at 30.0-fold ($\Delta\Delta Ct = -4.91$, $\log_2 FC = 4.91$, $p = 0.001$), and PER12 at 15-fold ($\Delta\Delta Ct = -3.91$, $\log_2 FC = 3.91$, $p = 0.003$). These results pointed to a strong activation of antioxidant defense systems in response to hyperhydric stress. Ethylene signaling and associated metabolic genes were also significantly upregulated: COX2 and ACO1 showed 35.0-fold ($\Delta\Delta Ct = -5.13$, $\log_2 FC = 5.13$, $p = 0.001$) and 28-fold ($\Delta\Delta Ct = -4.81$, $\log_2 FC = 4.81$, $p = 0.001$) increases respectively, while ETR1 and ACS11 were upregulated by 22-fold ($\Delta\Delta Ct = -4.46$, $\log_2 FC = 4.46$, $p = 0.001$) and 18-fold ($\Delta\Delta Ct = -4.17$, $\log_2 FC = 4.17$, $p = 0.002$). Two genes were identified as statistically non-significant expressions: ADH12 had a modest 1.8-fold increase ($\Delta\Delta Ct = -0.85$, $\log_2 FC = 0.85$, $p = 0.156$), and GMP exhibited a 1.7-fold increase ($\Delta\Delta Ct = -0.77$, $\log_2 FC = 0.77$, $p = 0.089$), indicating that these genes were not consistently altered under hyperhydric condition in this medium. In semi-solid medium, the gene expression trends were remarkably consistent with those observed in liquid medium. Again, eight out of ten genes were significantly upregulated in hyperhydric shoots (Cv1-HHS) compared to silicon treated shoots (Cv1-SiS). The antioxidant genes CAT1, APX, and SOD each showed identical maximum expression of 35-fold ($\Delta\Delta Ct = -5.13$, $\log_2 FC = 5.13$; $p = 0.001$ for CAT1 and APX, and $p = 0.002$ for SOD), while PER12 maintained significant upregulation at 12-fold ($\Delta\Delta Ct = -3.58$, $\log_2 FC = 3.58$, $p = 0.001$). Ethylene related genes also maintained robust expression: COX2 was upregulated by

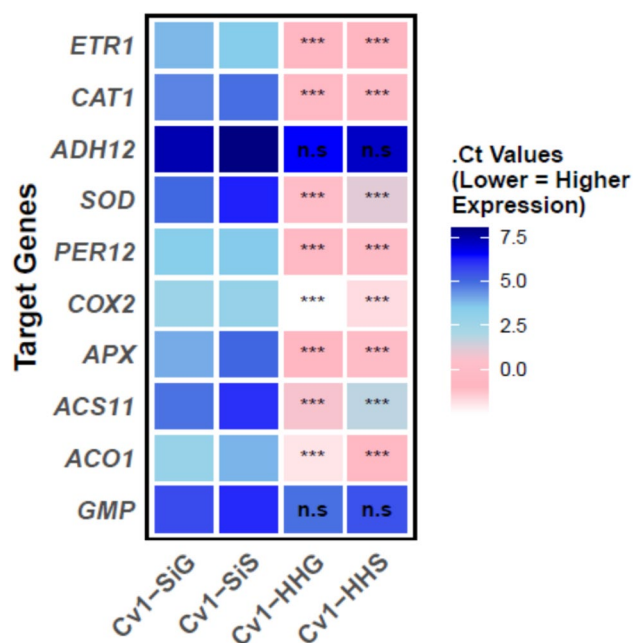


Figure 5. Heatmap representation of relative gene expression levels (Ct values) of hyperhydricity related genes in lingonberry (*Vaccinium vitis-idaea* L.) shoots under hyperhydric and silicon treated conditions in two types of media (liquid and semi-solid). Gene expression was analyzed using RT-qPCR across four groups: silicon treated shoots in Growtek (liquid) (Cv1-SiG) and Sigma jar (semi-solid) (Cv1-SiS) media, and hyperhydric shoots in liquid (Cv1-HHG) and semi-solid (Cv1-HHS) media. The color gradient represents Ct values, where darker blue indicates lower expression and lighter pink or red indicates higher expression. Genes associated with oxidative stress (for example CAT1, SOD, APX), ethylene signaling (ETR1, ACO1, ACS11), and metabolic regulation (COX2, PER12) were significantly upregulated ($p \leq 0.05$, ANOVA with Tukey's HSD). Non-significant differences (n.s.) were observed for ADH12 and GMP, suggesting limited or variable response under hyperhydric conditions.

28-fold ($\Delta\Delta Ct = -4.81$, $\log_2 FC = 4.81$, $p = 0.001$), ACO1 by 25.0-fold ($\Delta\Delta Ct = -4.64$, $\log_2 FC = 4.64$, $p = 0.001$), ETR1 by 21-fold ($\Delta\Delta Ct = -4.39$, $\log_2 FC = 4.39$, $p = 0.001$), and ACS11 by 20-fold ($\Delta\Delta Ct = -4.32$, $\log_2 FC = 4.32$, $p = 0.003$). Genes that were non-significant in liquid medium also showed non-significant expression changes in semi-solid medium as well, ADH12 had a 1.9-fold increase ($\Delta\Delta Ct = -0.93$, $\log_2 FC = 0.93$, $p = 0.142$) and GMP a 1.6-fold increase ($\Delta\Delta Ct = -0.68$, $\log_2 FC = 0.68$, $p = 0.093$). The results obtained showed consistent and significant transcriptional reprogramming in hyperhydric tissues across both liquid and semi-solid media, with 80.0% of genes showing statistically significant upregulation in both conditions. The gene expression patterns, particularly the strong activation of antioxidants and ethylene signaling pathways, suggested a conserved and medium independent molecular response to hyperhydric stress. Genes, such as CAT1, APX, SOD, ACO1, and COX2, emerged as key transcriptional markers

of hyperhydricity, while ADH12 and GMP remained stable, indicating their limited role under the tested conditions (Fig. 5).

Discussion

Hyperhydricity (HH) is a serious challenge in plant tissue culture, leading to abnormal water accumulation, distorted growth, and reduced plant quality. This study was the first comprehensive investigation of hyperhydricity in micropropagated *Vaccinium vitis-idaea* L. (lingonberry), offering detailed insights into the structural, biochemical, and molecular responses associated with HH and highlighting the beneficial effects of silicon (K_2SiO_3) treatment for recovery.

Morphologically, hyperhydric shoots grown in both liquid and semi-solid media displayed typical HH symptoms, including thickened, brittle stems, translucent curled leaves, and, in severe cases, necrotic tissue. Water content was particularly high in Cv1-HHG (86%) and Cv1-HHS (77%). The present study findings aligned with those of Barua *et al.* (2025), who reported that hyperhydricity in *Vaccinium* species (blueberry) caused structural disorganization at both the cellular and tissue levels, particularly under Growtek liquid-culture conditions. Barua *et al.* (2025) also observed that the higher humidity and nutrient availability in liquid medium exacerbate water uptake, triggering hyperhydric effects. Notably, both studies reported an increased accumulation of phytochemicals, such as catechins, and other phenolics in hyperhydric tissues. This suggested a shared stress induced metabolic shift in response to HH, possibly as part of the plant's defense mechanism. However, after the application of 1.2 mM K_2SiO_3 , water content significantly declined to 63% and 61.6%, respectively, by wk 8. Clear signs of shoot regeneration were observed, including healthier leaf structure and elongated internodes by the 16th wk. This progression underscored the effectiveness of silicon in promoting structural and physiological recovery, consistent with earlier findings in carnation and other stressed plants (Mali and Aery 2008; Soundararajan *et al.* 2017). Previous studies in related *Vaccinium* species, such as blueberry, support the current findings. Gao *et al.* (2018) reported that hyperhydric blueberry plantlets exhibited disrupted leaf and vascular anatomy, high water content, and stomatal dysfunction, all contributing to poor gas exchange and water regulation. Similarly, Polivanova and Bedarev (2022) emphasized that HH disrupts both gas exchange and photosynthesis, primarily through anatomical changes in leaf structure, cell wall thinning, and chloroplast degradation, which mirror the morphological and ultrastructural

symptoms observed in the present study's lingonberry cultures. Anatomical analysis using SEM confirmed that the structural disruptions caused by HH epidermal cells were irregular, stomata were malformed, and trichomes were distorted. Vascular tissues showed loose organization with fragile walls and enlarged intercellular spaces, all contributing to impaired water and nutrient movement. After silicon treatment, leaf and stem tissues exhibited restored epidermal integrity, reformed trichomes, compact vascular bundles, and reduced intercellular spaces. These results highlighted silicon's role in reinforcing tissue structure and functional recovery (Ma and Yamaji 2006; Zuccarini 2008). Phytochemically, UHPLC-MS/MS revealed that HH triggered overaccumulation of several secondary metabolites. Cyanidin-3-glucoside increased to 21.83 mg kg⁻¹ FW in Growtek and 19.46 mg kg⁻¹ in Sigma hyperhydric shoots, while procyanidin B2 reached 169.93 mg kg⁻¹ FW. Other notable increases included quercetin (18.22 mg kg⁻¹), rutin (14.55 mg kg⁻¹), and chlorogenic acid (767.91 mg kg⁻¹). These compounds are known as antioxidants, and their accumulation likely reflected a defensive response to oxidative stress. The overaccumulation of secondary metabolites in HH lingonberry shoots, including cyanidin-3-glucoside, chlorogenic acid, and flavonols, further supports the hypothesis that HH induces a strong oxidative stress response. Similar trends have been documented in carnation, where ROS accumulation upregulated antioxidant defense enzymes (SOD, CAT, and APX) and altered protein expression involved in primary metabolism and stress signaling (Muneer *et al.* 2016). Following silicon treatment, levels of these metabolites dropped significantly; cyanidin-3-glucoside reduced to 11.66 mg kg⁻¹, procyanidin B2 to 97.24 mg kg⁻¹, and chlorogenic acid to 256.10 mg kg⁻¹, suggesting that silicon helps modulate metabolic stress responses (Manivannan *et al.* 2016).

On the molecular level, gene expression analysis showed strong upregulation of eight out of ten genes in hyperhydric tissues across both media types. Antioxidant related genes, like *CAT1*, *APX*, and *SOD*, showed more than 30-fold increases, while ethylene related genes, such as *ACO1*, *ACS11*, *COX2*, and *ETR1*, were also highly elevated. These patterns were consistent with earlier reports showing that hyperhydricity is driven by oxidative and hormonal stress pathways (Gao *et al.* 2022; Polivanova and Bedarev 2022). Notably, *ADH12* and *GMP* did not show major expression changes, indicating they may play a limited role in HH under these conditions. Importantly, silicon treatment downregulated the highly expressed genes, confirming its role in rebalancing stress responses at the transcriptional level. The present study demonstrated that silicon application significantly promoted the recovery of hyperhydric

micropropagated shoots, aligning with previous research that underscored silicon's capacity to modulate physiological and molecular responses under stress conditions (Soundararajan *et al.* 2017). Notably, silicon downregulated the expression of stress associated genes, including those involved in ethylene biosynthesis, thereby indicating its role in restoring hormonal homeostasis and cellular integrity. This transcriptional regulation suggested that silicon acts as a crucial signaling modulator, rebalancing stress responses at the gene expression level (Soundararajan *et al.* 2017). Several prior studies have established links between reactive oxygen species (ROS) accumulation and ethylene production in hyperhydric tissues. Gao *et al.* (2018) and Polivanova and Bedarev (2022) proposed that increased ROS levels can enhance ethylene biosynthesis and exacerbate hyperhydric symptoms. The present findings supported this model, as silicon treatment reduced ROS levels, evidenced by decreased antioxidant enzyme activities, and simultaneously suppressed ethylene related gene expression. This dual action indicated that silicon effectively disrupts the ROS ethylene levels, which is fundamental to hyperhydricity persistence. Moreover, silicon strengthened plant tissues by being deposited in cell walls and cuticles, making them more rigid and less permeable. This helps reduce unwanted water absorption, a key feature of hyperhydricity (Ma and Yamaji 2006). Its role in stabilizing cellular membranes and water relations further contributed to physiological stabilization, enabling tissues to recover from vitrification like states. Additionally, proteomic analyses (Soundararajan *et al.* 2017) revealed that silicon modulates proteins involved in hormone signaling, stress response, and metabolism, reinforcing its role at multiple regulatory levels. By adjusting these pathways, silicon not only alleviated oxidative stress but also promoted cellular differentiation and normal growth patterns. In conclusion, this first-of-its-kind study in lingonberry micropropagation confirmed that hyperhydricity arises from a combination of water imbalance, oxidative stress, and hormonal dysregulation, resulting in extensive morphological and physiological damage. However, treatment with 1.2 mM K_2SiO_3 proved highly effective in restoring normal growth, tissue structure, and gene expression. These findings provided a valuable framework for understanding and managing hyperhydricity, with practical applications in improving the quality and success of *in vitro* propagated lingonberry and potentially other woody species.

Conclusion

The morphological, anatomical, biochemical, and molecular abnormalities linked to hyperhydricity in micropropagated *Vaccinium vitis-idaea* L. were highlighted in this study, which offered the first comprehensive understanding of this physiological condition. Excessive water accumulation, structural abnormalities, and strong oxidative and hormonal stress responses were the hallmarks of hyperhydricity, which negatively impacted plant quality. By downregulating stress responsive genes, restoring normal tissue architecture, and rebalancing secondary metabolite buildup, the application of 1.2 mM potassium silicate (K_2SiO_3) successfully reduced these symptoms. These results showed that silicon supplementation is a viable method of controlling hyperhydricity in lingonberries and maybe other woody species, opening the door to better *in vitro* propagation techniques of this valuable nutraceutical crop.

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Authors 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; S.M and R.B assisted in data collection for water content, and finalizing the manuscript. H.D.M. and A.U.I. 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 interest The authors declared that they have no competing interests.

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