



Exploring two bioreactor systems for micropropagation of *Vaccinium membranaceum* and the antioxidant enzyme profiling in tissue culture-raised plants

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Abstract

Vaccinium membranaceum (black huckleberry) is known for its high content of bioactive compounds. This study introduces a novel approach for bioreactor micropropagation using stationary (Growtek) and temporary immersion (RITA) bioreactor systems using a liquid nutrient medium to enhance the in vitro propagation of black huckleberry. Adventitious shoot regeneration from leaf explants reached 80% efficiency in a semi-solid nutrient medium with 10 μM thidiazuron. Shoot proliferation was optimized in liquid culture, where the Growtek system yielded the most robust shoot regeneration at 76% and second highest was observed on RITA at 40.33%. Morphological and histological analyses of different cultured explants revealed various stomatal density, trichome formation and mesophyll tissue organization. Biochemical profiling of antioxidant enzymes showed that greenhouse grown leaves had higher levels of bioactive compounds, such as flavonoids, proanthocyanins, and antioxidant activity. However, Growtek-cultured leaves exhibited elevated phenolic content and catalase activity. Additionally, antioxidant enzymes, including catalase ($2.8 \text{ U g}^{-1} \text{ min}^{-1} \text{ fw}$) and superoxide dismutase ($0.80 \text{ U g}^{-1} \text{ min}^{-1} \text{ fw}$), were higher in Growtek and leaf-cultured shoots, respectively. The greenhouse grown plants demonstrated the highest overall phytochemical activity, with the most abundant proanthocyanins ($8.3 \text{ mg g}^{-1} \text{ fw}$) and anthocyanins ($26 \text{ mg g}^{-1} \text{ fw}$). This work presents a highly efficient liquid micropropagation protocol for black huckleberry with insights into its bioactive compounds and antioxidant enzyme profiles, highlighting its potential in commercial production and medicinal applications.

Keywords Bioreactor micropropagation · Thidiazuron · ZEA · Bioactive compound · SEM · Histology

Introduction

The increasing global demand for nutraceutical-rich crops has heightened interest in cultivating small fruit crops such as *Vaccinium membranaceum* (*V. membranaceum*) commonly known as black huckleberry. It is renowned for its high content of bioactive compounds (Martău et al. 2023 ; Precup et al. 2022). These berries are a rich source of flavonoids, phenolic acids, anthocyanins, and antioxidants, all of which contribute to various health benefits, including anti-diabetic, anti-inflammatory, and antibacterial activities, along with its free radical scavenging properties (Lee et al. 2004a). Furthermore, black huckleberries provide essential minerals, including calcium, magnesium, zinc, and iron, enhancing their nutritional profile (Kuhnlein 1990; Lee et al. 2004b; Taruscio et al. 2004).

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V. membranaceum, a member of the Ericaceae family, thrives in the mountainous regions of North America, particularly in the Pacific Northwest and Rocky Mountains (Richards and Alexander 2006). These habitats range from mid-alpine regions at altitudes of up to 3500 m (11,500 feet) above sea level to mountain slopes, forests, and lake basins (Barney et al., 2007; Gorzelak et al. 2012; Pervin et al. 2013). Despite its growing commercial value, the large scale propagation of black huckleberry is constrained by challenges such as low seed germination rates, poor root formation, and high phenolic content, which hampers traditional propagation methods (McKechnie et al. 2009). In vitro micropropagation offers a promising alternative to address these challenges regarding year-round production and efficient propagation of high-value plants like huckleberry (Debnath and Barney 2012).

Micropropagation, particularly through liquid-based bioreactor systems, offers several advantages over conventional semi-solid media. Bioreactors, which include stationary and temporary immersion systems, provide enhanced shoot proliferation, faster growth, and improved nutrient uptake due to better contact with the liquid medium (Debnath 2017). Previous studies have demonstrated the effectiveness of bioreactors in other *Vaccinium* species, though they have not been applied to black huckleberry. The use of growth regulators such as thidiazuron (TDZ) and zeatin (ZEA) in micropropagation further promotes efficient shoot regeneration and plantlet development (Ghosh et al. 2018; Sharma et al. 2023; Sikdar et al. 2021).

This study aims to establish a novel protocol for adventitious shoot regeneration and plant formation for the first time in black huckleberry in a liquid medium using bioreactor systems. The approach involves initial regeneration on a semi-solid medium and shoot proliferation in a liquid medium using two distinct bioreactor systems. Furthermore, the bioactive properties of the regenerated plants were assessed to determine the influence of in vitro and *ex vitro* culture conditions on their bioactive components. The integration of liquid bioreactor micropropagation system, along with the evaluation of bioactive attributes, demonstrates promising potential for optimizing the large-scale commercial cultivation of black huckleberry.

Materials and methods

Plant material, culture condition and shoot regeneration

Greenhouse-grown plants of black huckleberry clone ‘VM31C’ maintained at St. John’s Research and Development Centre, Agriculture and Agri-Food Canada, St. John’s,

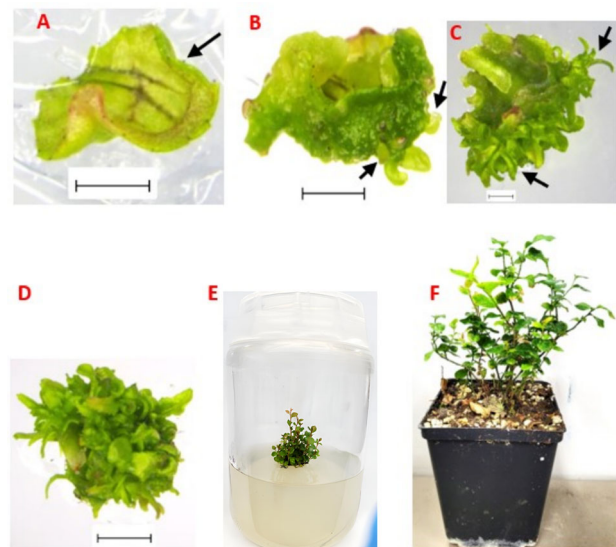


Fig. 1 Indirect organogenesis and plant formation from *Vaccinium membranaceum* leaves on a basal medium supplemented with different plant growth regulator concentration. **A** Leaf exposed to photoperiod after 2 weeks of inoculation. **B** Primordium development at 5 weeks. **C** Starting of bud formation at 8 weeks. **D** Adventitious shoots developed on every surface of leaf culture at 10 weeks (Bars = 0.5 cm). **E** Proliferated multiple shoots after 12 weeks. **F** One-year old individual plant derived from adventitious shoots

Newfoundland and Labrador, Canada (Debnath and Barney 2012) were used in this study. The black huckleberry plant materials included in the study fulfil the appropriate guidelines and legislation at institutional, national, and international levels legislation (Debnath and Arigundam 2020; Debnath and Barney 2012; Sikdar et al. 2021). Young, actively growing leaves excised from greenhouse grown plants, were surface sterilized and cultured on a modified Debnath and McRae’s berry basal medium (BM) (Debnath and McRae 2001) containing 25 g L⁻¹ sucrose, 1.25 g L⁻¹ Gelrite (Sigma Chemical Co., St. Louis, MO, USA) and 3.5 g L⁻¹ agar (Sigma Chemical Co.), pH 5.0 following (Arigundam et al. 2020; Debnath and Arigundam 2020). Sterilized explants were cultured on 100 mm × 25 mm Petri plates with transparent lids (Fisher Scientific, Fair Lawn, NJ, USA). In this study, two plant growth regulators (TDZ, ZEA) were used. Each plate contained 25 mL of BM supplemented with either 0 (control), 2.0, 5.5, 8.5, and 10 μM of TDZ and ZEA separately. Three dishes were utilized for each treatment, each with five explants. The cultures were kept in the dark at 20 ± 2 °C for two weeks before being moved to cool-white, fluorescent lamps’ diffused light (PPFD at 30 μmol m⁻² s⁻¹, 16 h photoperiod) in a plant growth chamber (Conviron, Winnipeg, Manitoba, Canada) to check indirect organogenesis. Data on shoot regeneration were collected after a culture period of 4 weeks established in *Vaccinium* species. Regenerated shoots (Fig. 1E) at 12 weeks were transferred to the

same medium containing 35 mL of BM (Debnath and McRae 2001) supplemented with 4.0 μM ZEA to promote further growth and maturation in a Sigma bottle (Ghosh et al. 2018; Sharma et al. 2023; Sikdar et al. 2021).

Shoot proliferation in semi-solid and liquid medium using stationary and temporary immersion bioreactor systems

Twelve-weeks-old nodal explants regenerated from leaves were used for proliferation on a semi-solid medium in Sigma bottle enriched with 4 μM ZEA and sub-cultured every 8 weeks (Debnath and Barney 2012). Proliferated shoots were also transferred into two different types of bioreactors: temporary immersion bioreactor (RITA; volume: 0.9 L; height: 13.5 cm; diameter: 13.5 cm; VITROPIC, Saint-Mathieu-de-Trévières, France) (Teisson and Alvard 1995; Arigundam et al. 2020) and stationary bioreactor (Growtek; Bel-Art™ SP Scienceware™ Growtek™ culture vessels, volume: 1.178 L; height: 15 cm; diameter: 5.7 cm; Fisher Scientific, Ottawa, Ontario, Canada) containing 200 mL of same but liquid medium (Arigundam et al. 2020; Sharma et al. 2011). The liquid medium in the bioreactors was supplemented with ZEA (4 μM) and served as a control without any plant growth regulators (Debnath and Barney 2012; Ghosh et al. 2018; Sharma et al. 2023; Sikdar et al. 2021). The Growtek bioreactor features a rotating explant holder, a medium-exchange side tube, and a polycarbonate casing. In contrast, the RITA bioreactor consists of cylindrical tanks, 15 cm tall, split into two parts: the top section holds plant material on a 1 cm polyurethane filter, while the bottom section holds the growth medium. An automated air pump creates overpressure in the lower section, causing the medium to flow through the filter into the upper part. An automated air pump creates overpressure in the lower section, causing the medium to flow through the filter into the upper part. The excess pressure released through an air vent in the lid, and the air pump's timer controls the duration and frequency of liquid immersion for the plant material, which is typically set at 15 min every 4 h. These cultures were maintained in a growth chamber with the same photoperiod, light intensity, and temperature conditions as those used for the initial shoot regeneration experiments. Each bioreactor vessel contained eight explants, and sub-culturing was performed every 8 weeks. Four vessels were assigned to each treatment, and this experiment was repeated three times (Debnath and Arigundam 2020).

Rooting and acclimatization

Shoots measuring 5–6 cm in length with 7–10 leaf were obtained from both node and leaf cultures of black huckleberry plants. After 12 weeks of in vitro culture period, these

shoots were maintained in green house. This greenhouse utilized plastic containers filled with a mixture of 2 parts peat and 1 part perlite (volume/volume). The samples were carefully removed from the agar in the sigma containers, taken out from the liquid medium of Growtek and RITA vessels, and subsequently rinsed with sterile water. Samples grown in a liquid medium were similarly cleansed with sterile water and displayed no solid residue. Shoots with 39.4 mM of Indole-3-butyric acid powder transferred into plastic container with relative humidity of 85%, a temperature of at $20 \pm 2^\circ\text{C}$, and a 16-h photoperiod were kept constant in the growth chamber (Convion, MTR30, Canada) for experimental purpose. A $90 \mu\text{mol m}^{-2} \text{s}^{-1}$ maximum photosynthetic photon flux (PPF) was maintained (Arigundam et al. 2020; Debnath and Arigundam 2020).

Scanning electron microscope (SEM) study

Scanning electron microscope studies were performed in shoot regeneration experiments to verify the nature of organogenesis following (Picoli et al. 2008a, b) and (Gao et al. 2018). The stigma like arrangement of bud formation hardly explained to the naked eye. Therefore SEM was employed to provide a more comprehensive and detailed visualization of the intricacies of bud formation. The texture and spatial arrangement of stomata with their epidermal cell condition on the leaf were analysed using SEM. Leaf samples that were 6 weeks old were collected from semi solid, Growtek, RITA, and greenhouse grown plants. The leaves were cut into 1 cm^2 pieces and then the samples were soaked for 24 h in 0.1 M phosphate buffer (pH 7.2) containing 2.5% glutaraldehyde (v/v), pH 7.0 at 4°C overnight. Samples were then washed with sample buffer three times and dehydrated with ethanol. The leaf samples were coated with gold sputtering. After coating with the thin layer (20 nm) of gold, the materials were then examined for SEM analysis using a FEI MLA 650FEG (FE-SEM, Ontario, Canada) to photograph the pictures after the ultramicrotomy were stained.

Histological investigation

To analyze cellular structures, including palisade parenchyma and spongy parenchyma in leaf tissues: the organization of mesophyll tissue, and to examine the arrangement and integrity of vascular tissues, such as xylem and phloem; histology is critical for assessing tissue differentiation and developmental physiology under various conditions. Six weeks old leaf samples from Sigma, Growtek, RITA and greenhouse grown plants and eight weeks old callus from leaf samples were placed in an FAA solution (formalin 37%, acetic acid glacial, ethanol 70%; 1:1:18 v/v) for 48 h at room temperature. These fixed samples were processed using a Tissue-tech VIP Tissue

Processor for critical drying and wax infiltration. The tissues were embedded in wax and cut into 5 or 10 mm sections using a rotary microtome (model Leica RM2135). These sections were floated on a water bath at about 40 °C to flatten them and then placed on slides. Slides were labeled with section order, thickness, and plant sample code, then affixed by melting the wax on a hot plate at 60 °C for 12 h. The slides were oven-dried at 38 °C, de-waxed, stained, mounted and observed using Lumenera Infinity camera. Histology is utilized for leaf and in vitro shoot visualization to analyze cellular structures, including parenchyma and spongy parenchyma in leaf tissues, and to examine the arrangement and integrity of vascular tissues, such as xylem and phloem, in stem sections. This technique allows for detailed anatomical observations critical for assessing tissue differentiation and developmental anomalies under various conditions (Almeida et al. 2012).

Biochemical analysis

Preparation of leaf extracts

Fresh leaves from regenerated shoots and proliferated shoots cultured in both semi solid and liquid media, under two bioreactor systems, were collected after six weeks to analyze the bioactive compounds. In brief, 500 mg of fresh leaves from each sample were homogenized in a FastPrep-24 Tissue and Cell Homogenizer (MP Biomedicals, Irvine, CA, USA) in 80% aqueous acetone containing 0.2% formic acid 1:4 (g:mL) to create leaf powder (Ghosh et al. 2018; Goyali et al. 2015). The homogenate was shaken at 4 °C for 30 min. Using an Allegra 64R (Beckman Coulter Inc., Palo Alto, CA, USA), the solution was centrifuged at 4 °C for 15 min at a speed of 13,000 rpm. The supernatant was collected to measure the levels of proanthocyanidin (PAC), total phenolic content (TPC), total antioxidant activity, glutathione reductase (GR), total flavonoid content (TFC), and anthocyanins except catalase (CAT) and superoxide dismutase (SOD).

Total flavonoid content (TFC)

The total flavonoid content of leaf extracts was measured using a colorimetric test (Bhatt and Debnath 2021; Goyali et al. 2015). 150 mL of 5% (wt/vol) sodium nitrite and 500 µL of catechin extract or standard solutions of catechin were added to 2 mL of distilled water (dH₂O). After five minutes, 150 µL of 10% (wt/vol) aluminum chloride (AlCl₃) was added. 1 mL of a 1 M sodium hydroxide solution was mixed after 6 min. Finally, 1.2 mL of dH₂O was mixed to dilute it. At 510 nm, both the mixture's (pink-colored) and the blanks' absorbance were measured. The measuring unit of TFC of leaves is milligrams of catechin equivalents per gram of leaves (Bhatt and Debnath 2021).

Total antioxidant activity

The scavenged free radicals were calculated as a percentage of radical inhibition. The leaf extracts and the standard (gallic acid) evaluation were based on the synthetic DPPH (2,2-diphenyl 1-picrylhydrazyl) radical's ability to scavenge free radicals. 1.7 mL of 0.06 mM DPPH methanolic solution was mixed with an aliquot (100 µL) of the diluted extract or gallic acid standard solution (5 µg mL⁻¹; ≥ 98% purity) (Bhatt and Debnath 2021). A blank solution comprised 80% aqueous acetone and stored in the dark for 45 min. Using an Ultrospec 4300 Pro UV–Visible spectrophotometer (Amersham Biosciences Corp., San Francisco, CA, USA), the reduction in absorbance was measured at 517 nm. The linearity of the gallic acid standard curve ($r^2 = 0.98$) was obtained in the range of 20–80 µg mL⁻¹. Following formula was used to determine percent inhibition (Bhatt and Debnath 2021):

Inhibition of DPPH activity as a percentage (%)

$$= \left[(A - B) / A \right] \times 100$$

where A represents the blank's optical density and B represents the leaf extract's optical density. The amount of antioxidant activity was calculated in milligrams of gallic acid equivalents per gram of leaves in the extract.

Antioxidant enzymes

With a few minor modifications, the extractions for CAT and SOD were carried out as described earlier (Sun et al. 2018). Leaf samples (0.5 g) were homogenized on ice in 4 ml of ice-cold buffer containing 50 mM Tris–HCl (pH 8.0), 15 mM (dithiothreitol) DTT, and 0.1 M sucrose. The homogenates underwent a 30-min, 13,000-g centrifugation at 4 °C. Supernatants were employed to measure the activity of enzymes and were kept at – 80 °C.

Catalase (CAT)

0.05 M phosphate buffer (pH 7) containing 3% H₂O₂ (w/w) was used as the reaction buffer. 20–100 µl of plant extract were added to the 900–980 µl reaction buffer solution. The activity was expressed in U (µmol) g⁻¹ (protein) min⁻¹ and the absorbance was measured at 240 nm for 3 min. The definition of unit (U) activity is 1 µmol of H₂O₂ degraded every minute. The equivalent CAT activity per mg of protein was calculated using the molar extinction value for H₂O₂ (43.6 M⁻¹ cm⁻¹).

Superoxide dismutase (SOD)

The activity of the SOD procedure is based on the spectrophotometric assessment of the inhibition of nitroblue tetrazolium (NBT) photochemical reduction at 560 nm. Leaf extract (30–100 μ l) and the assay buffer (900–970 μ l) were mixed which includes 0.1 M potassium phosphate buffer (pH 7.), 10 mM ethylene diaminetetraacetic acid (EDTA), 4.2 mM NBT, 260 mM methionine, 130 μ M riboflavin. At a light intensity of 300 μ mol m⁻² s⁻¹, reactions were run for 10 min, and the absorbance at 560 nm was determined. The absorbance at 560 nm of the non-irradiated reaction mixture served as the control, from which the absorbance of the irradiated sample was subtracted. One unit of SOD was defined as the quantity of enzyme that caused 50% inhibition of NBT reduction during the experiment, and the findings were reported per mg of protein (Sun et al. 2018).

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

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 (Samad et al. 2014).

Glutathione reductase (GR)

The activity of GR was measured spectrophotometrically at 340 nm for 3 min, starting from the rate of NADPH to NADP conversion ($\epsilon_{340} = 6.2 \text{ m M}^{-1} \text{ cm}^{-1}$). The combination contains 20–100 ml of extract and 980–900 μ l of 50 mM HEPES buffer (pH 8.0) containing 0.5 mM EDTA and 0.25 mM NADPH. The GSSG was added to the process at a final concentration of 1 mM, and the absorbance at 340 nm was measured. The equivalent GR activity per mg of protein was calculated using a molar extinction coefficient value of $6.2 \text{ m M}^{-1} \text{ cm}^{-1}$ (Wang et al. 2017; Sikdar et al. 2021).

Proanthocyanin content

The concentration of proanthocyanidins (condensed tannins) in leaf extracts was measured using a modified vanilla spectrophotometric method, with a molecular extinction value of 0.003 CE mg g⁻¹ fresh leaf weight (Sikdar et al. 2021). In 0.5 mL of diluted extracts and standard (catechin) solutions; 2.5 mL of vanillin-HCl reagent (0.5%) was added and mixed well. For 20 min, this mixture was incubated in the dark at 30 °C. Compared to the standard blanks, the absorbance at 500 nm was calculated. The benchmark for proanthocyanidin expression is catechin, with a concentration range of 50 to 500 m (Debnath and McRae 2001; Sikdar et al. 2021). Catechin standard curve was created using 0–2.5 mg ml⁻¹

of absorbance. The amount of proanthocyanidin per gram of fresh leaf was weighted in milligrams of catechin equivalents.

Total phenolic content

Folin-Ciocalteu reagent, an acidic phosphomolybdotungstate solution that oxidizes phenolates and yields a blue pigment, is used to determine the total phenolic content of leaf extracts (Bhatt and Debnath 2021). 100 μ L of properly diluted sample extracts were mixed with 200 μ L of saturated sodium carbonate and 100 μ L of the Folin-Ciocalteu reagent and then added 1.5 mL of distilled water. The mixture was centrifuged at 4000 g for 10 min in an Allegra 64R after leaving it in the dark and at room temperature for 35 min. Using a spectroscope and the standard blanks, the absorbance was determined at 725 nm. The results was given as mg GAE g⁻¹ flw. The absorbance values were calculated linearly from the standard calibration curve ($r^2 = 0.98$) using the gallic acid standard solution (5 mg/mL; $\geq 98\%$ purity) in the range of 2.5–10 μ g ml⁻¹. The total phenolic contents were measured in milligrams of gallic acid equivalents per gram of leaves using gallic acid molar coefficient extinction value $5.2 \text{ M}^{-1} \text{ cm}^{-1}$ as the standard (Bhatt and Debnath 2021).

Anthocyanin assay

The pH differential approach (Goyali et al. 2015) was used to quantify the monomeric anthocyanin concentration of the plant extract with only minor adjustments. The standard (cyanidin-3-glucoside) and two aliquots of the sample extract were diluted, 2.5 ml 0.025 M KCl (pH 1.0) and 2.5 ml of 0.4 M sodium acetate (pH 4.5) were poured respectively in different tubes containing 0.5 ml aliquots of distilled water. The mixtures were incubated in the dark at room temperature for 20 min. The absorbance of each combination was assessed at 510 nm and 700 nm using an ultraviolet spectrophotometer. The following formula was used to determine the total anthocyanin content:

AnthocyaninContent

$$(\text{mg L}^{-1}) = \left(A \times \text{MW} \times \text{DF} \times 1000 / (e \times l) \right)$$

where A (absorbance) = ($A_{\lambda 510} - A_{\lambda 700}$) pH 1.0 – ($A_{\lambda 510} - A_{\lambda 700}$), MW (molecular weight) = 449.2 g mol⁻¹ for cyanidin-3-glucoside; DF = dilution factor; e = 26,900 L mol⁻¹ cm⁻¹ for cyanidin-3-glucoside; and l = path length in cm. The concentration of all anthocyanin pigments in fresh leaf was represented as cyanidin-3-glucoside equivalents (C₃GE) in mg g⁻¹ using molar extinction 26.9 g mol⁻¹.

Statistical analysis

Statistical data were analysed using one-way analysis of variance (ANOVA), followed by Duncan's post hoc test with SPSS software (IBM SPSS). Multiple comparisons were further evaluated using Tukey's Honestly Significant Difference (HSD) test. Linear regression analysis was performed in RStudio (version 4.4.1 (2024-06-14 ucrt)). Results were expressed as mean $\pm$ standard error, with statistical significance set at $p \leq 0.05$. Media lacking TDZ and ZEA were excluded from evaluation due to the absence of significant results. Biochemical data, including correlation coefficients (r), coefficients of determination (r^2), and linear regressions for all bioactive compounds, were evaluated using JMP Pro 17.00 software (https://www.jmp.com/en_ca/home.html).

Results

Effect of different concentrations of TDZ and ZEA on callus formation and shoots regeneration

A different concentration of 2.0 μ M, 5.5 μ M, 8.5 μ M, 10 μ M of both TDZ and ZEA were used for the growth of young *V. membranaceum* leaves. Following placement on a semi-solid BM (Debnath and McRae 2001), dedifferentiation structures became visible after three weeks (Fig. 1A). By 5th week, cell division within the dedifferentiated cells formed primordial cells (Fig. 1B), with development occurring across the entire leaf surface. Bud formation was observed at 8th weeks, with shoot initiation from the meristem occurring around 10 to 11 weeks after inoculation. Once multiple shoots had developed from the leaves (Fig. 1D), they were transferred to a semi-solid BM (Debnath and McRae 2001) containing 4 μ M ZEA to promote plantlet development. After 12 weeks (Fig. 1E), the plants were acclimatized in greenhouse conditions in peat:perlite pots (Fig. 1F).

TDZ (10 μ M) and ZEA (8.5 μ M) were effective on shoot regeneration from leaf explants cultured on semi solid media when cultured in the dark for 2 weeks, followed by under cool-white, fluorescent lamps' diffused light. In the early stages of 2 weeks, the callus initially displayed a dark green colour (Fig. 1A), but after four weeks, it transitioned to a lighter green containing brown edges while growing on a semi-solid medium (Fig. 1B).

A higher percentage of explants formed callus on MS medium supplemented with 10 μ M TDZ hormone (Fig. 2A, B) in shoot regenerated 10 weeks on semi solid media. The medium fortified with 10 μ M TDZ concentration was the most optimal (75.7 ± 0.67) for stimulating callus formation; while at 8.5 μ M ZEA, significant callus formation was observed (64.3 ± 0.33). In contrast, TDZ concentrations of 2.0 μ M and 5.5 μ M was resulted in lower callus formation,

with values of 7.6 ± 0.33 and 11.3 ± 0.33 , respectively. Shoot bud regeneration was highest at 10 μ M TDZ (81.0 ± 0.58), while ZEA only moderately promoted callus formation at 8.5 μ M (50.7 ± 0.33) but had generally low shoot regeneration across all concentrations (Fig. 2a). Same as callus formation result, the number of shoots per explant was significantly highest at 10 μ M TDZ, yielding 14 shoots per explant, while lowest number recorded at 8.5 μ M ZEA (5 shoots per explant). Finally, TDZ was more effective than ZEA for both callus formation and shoot regeneration, particularly at higher concentrations (Fig. 2A, B). Regenerated shoots were observed after 12 weeks of culture (Fig. 1E).

Morphological analysis of proliferated shoots on a semi-solid and in a liquid medium under two bioreactor systems

Shoot regeneration proficiency refers to the ability of proliferated shoot (explant) to produce new shoots. The explants cultured in Growtek exhibited superior results in terms of proliferated shoot regeneration efficiency (76 ± 0.57) compared to explants cultured in RITA (40.33 ± 0.33). However, the maximum number of shoots per explant was observed in RITA (14.49 ± 0.56). Regarding leaf production (10.7 ± 0.15), shoot height (1.96 ± 0.03), and shoot vigor (7.33 ± 0.66), the highest results were obtained in explants cultured in Growtek system. Between all of the treatments, the variability was high in liquid bioreactor (Growtek) cultured explants and significantly higher in degree of freedom and mean square. In each test of semi solid, Growtek and RITA explants, F statistics value was numerically more in number than F-critic value, which is statistically significant ($P \leq 0.05$) (Table 1; Fig. 3).

SEM and Histology observation

Upon examining the callus under an electron microscope, irregular primordium initiation was observed after five weeks (as depicted in Fig. 4B). The primordial structure observed along with the incision showed globular nodule structures (Fig. 4A). With a scale bar of 200 μ m, these primordial originated from a callus. The redifferentiation process results in the generation of multiple shoots from a single leaf. Primordial formation was not found without hormone treatment; the leaves turned black or brown after 2 weeks of dark exposure.

Stomata in shoot proliferated leaves cultured in Sigma bottle exhibited an organized and small paracytic arrangement surrounding the opening between guard cells (Fig. 5A). Figure 5B displayed distorted leaf stomata with a smeared, thickened, and closed appearance. The abnormal growth due to under liquid media disrupted the plant's development.

The stomata of the hyperhydric plantlets exhibited diminished pilocytic stomatal density, malformed and elongated

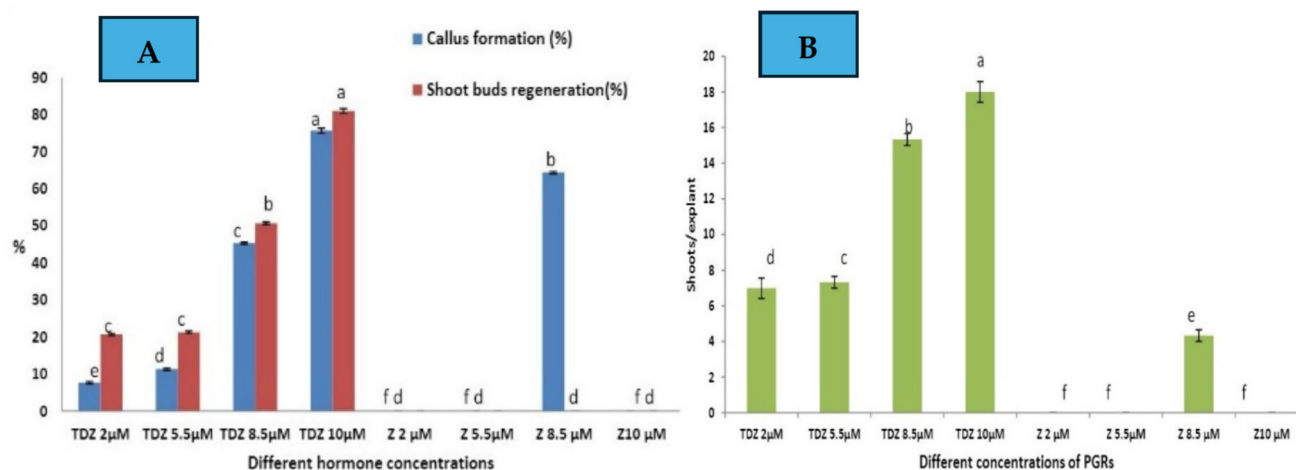


Fig. 2 Effect of plant growth regulators on **A** callus formation and adventitious bud regeneration and **B** percentage of shoots per explant were observed of *V. membranaceum* on a semi-solid medium at

12 weeks. Means $\pm$ Standard error according to Duncan's multiple range test at $P \leq 0.05$, whereby different letters depicted the significant differences ($n = 5$)

Table 1 Effect of semi-solid medium and different bioreactor systems on shoot proliferation

Culture condition	Shoot regeneration ^x	Shoot per explant (no.) ^x	Shoot Height (cm) ^x	Leaves per shoot (no.) ^x	Shoot vigour (range 1–8) ^x
Semi solid	59.33 $\pm$ 0.66 ^b	4.47 $\pm$ 0.18 ^c	1.89 $\pm$ 0.06 ^a	9.17 $\pm$ 0.16 ^b	5.33 $\pm$ 0.88 ^a
Growtek	76 $\pm$ 0.57 ^a	9.44 $\pm$ 0.35 ^b	1.96 $\pm$ 0.03 ^a	10.7 $\pm$ 0.15 ^a	7.33 $\pm$ 0.66 ^a
RITA	40.33 $\pm$ 0.33 ^c	14.49 $\pm$ 0.56 ^a	1.66 $\pm$ 0.02 ^b	9.7 $\pm$ 0.15 ^b	5.67 $\pm$ 0.33 ^a

^xValues are means $\pm$ standard error. According to Duncan's multiple range test, values within the same column that share identical letters are not significantly different at the 5% significance level. Data were collected after 8 weeks of culture

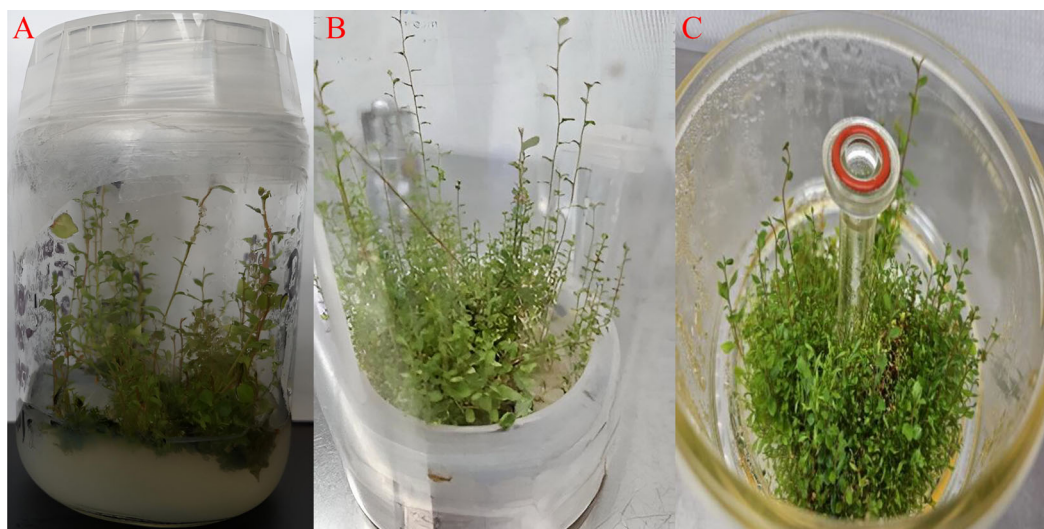


Fig. 3 Different culture conditions: stem parts transferred to **A** semi solid cultured bottle, **B** Growtek vessel and **(C)** RITA vessel with proper sterilization procedure containing ethanol, clorox and sterile distilled

water followed by Debnath method (Debnath 2009a) and all rooted shoots transferred to small pot with proper perlite and peat (same as Fig. 1F) followed by Sikdar et al. (Sikdar et al. 2021)

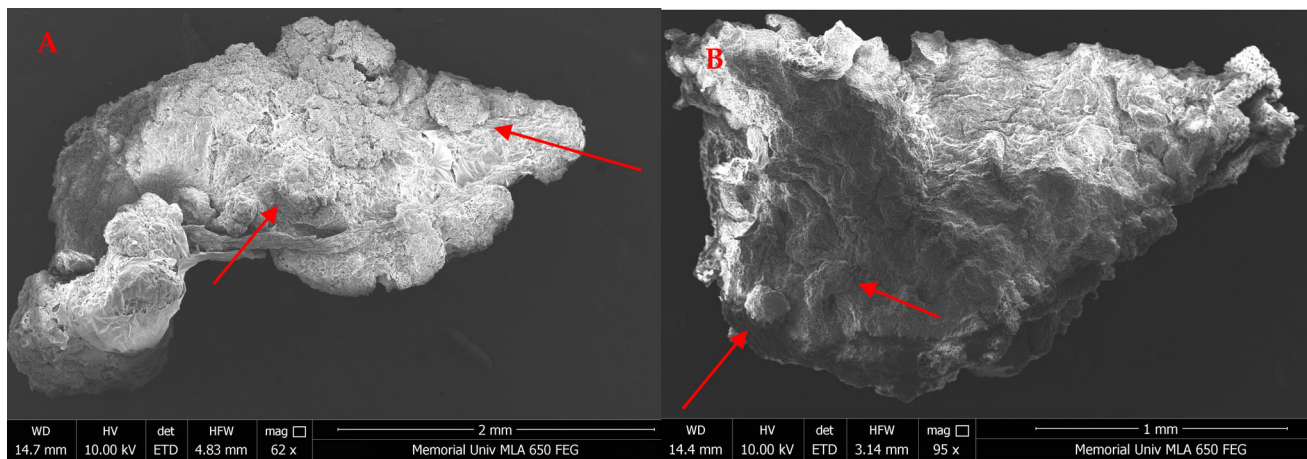


Fig. 4 Indirect organogenesis from leaf of *V. membranaceum* showed bud formation from callus observe on BM supplemented with different hormone. **A** Initiation of primordium development of *V. membranaceum*

callus at 5 weeks supplemented with $8.5 \mu\text{M}$ ze (bar 2 mm); **B** Some primordium development to form globular nodule with $10 \mu\text{M}$ TDZ hormone in medium after 5 weeks (bar 1 mm)

epidermal trichomes along with closed stomata on their surface. Most stomata filled with water turned into hyperhydric conditions. Leaves grown in RITA vessels showed parallelly organized stomata appearance with compact epidermal cells (Fig. 5C). Plants with fewer stomata in leaves grown in an *ex vitro* (greenhouse) setting were less organized in epidermal cell (Fig. 5D), but there were fewer active stomata found in cultured using Semi solid media.

Spongy parenchyma shows loosely arranged cells with larger intercellular spaces. The vascular bundles (VB) are visible and the epidermal cells appear irregular in shape and size, with a slightly thickened cuticle layer and uneven stomatal distribution (Fig. 5E). Greenhouse (Fig. 5E) grown plants have been shown to organize both Palisade and spongy parenchyma, although the Growtek (Fig. 5F) bioreactor has altered this orientation. Cells are disorganized than in naturally grown plant's leaf. SP cells exhibit even larger air spaces and lack structural integrity due to high water content. Although the VB is slightly more prominent, the tissue morphology shows signs of thicker cell walls and fewer stomata (Fig. 5F). Cells of PP appear more elongated but are less compact, with noticeable vacuoles. The SP shows significant air spaces, indicating compromised gas exchange. The vascular system is visible but underdeveloped. The epidermal cells are irregularly shaped, and stomata are less frequent, with uneven distribution (Fig. 5H). The proliferated shoot's leaf under semi-solid media shows less defined PP than the RITA samples. The SP is compact, with fewer air spaces, indicating structural integrity. The VS is more distinct and The epidermis is regular, with well-formed stomata and a consistent cuticle layer, reflecting the plant's adaptation to the external environment (Fig. 5G). The undifferentiated tissue in leaf-derived callus cultures have high cell division activity with

dense cytoplasm and presence of small, meristematic-like cells (Fig. 5I).

Total flavonoid content and antioxidant activity quantification

One way ANOVA analysis is represented with an analysis of variance, means and standard deviation (with a significant value $p \leq 0.05$) for both flavonoid and antioxidant compounds measurement per leaves. The propagation methods in different culture environments with various hormones implemented into the media are the major factors in differentiating the amount of secondary metabolite contents per gram leaf. For both flavonoid and antioxidant activity, the amounts of flavonoid compounds in *V. membranaceum* showed in this descending order: Green House ($0.09 \text{ mg GAE g}^{-1} \text{ flw}$ (fresh leaf weight)) > Growtek culture ($0.05 \text{ mg GAE g}^{-1} \text{ flw}$) > RITA culture ($0.05 \text{ mg GAE g}^{-1} \text{ flw}$) > Sigma culture ($0.05 \text{ mg GAE g}^{-1} \text{ flw}$) > Leaf Culture $0.007 \text{ mg GAE g}^{-1} \text{ flw}$ (Fig. 6A). The results of antioxidant activity per gram of leaves are: Green House ($0.9 \pm 0.05 \text{ CE g}^{-1} \text{ flw}$) > Growtek culture ($0.4 \pm 0.06 \text{ CE g}^{-1} \text{ flw}$) > RITA culture ($0.4 \pm 0.04 \text{ CE g}^{-1} \text{ flw}$) > Leaf culture ($0.4 \pm 0.02 \text{ CE g}^{-1} \text{ flw}$) > Sigma culture ($0.4 \pm 0.1 \text{ CE g}^{-1} \text{ flw}$) (Fig. 6B). The maximum amount was obtained by plants grown in greenhouses for both compounds. All tissue-cultured plants have the same quantity of flavonoids, although the component amounts vary slightly. In antioxidant activity, the difference between the two highest amounts of Growtek plants and greenhouse plants is 0.4, or twice as of Growtek plant as greenhouse plant.

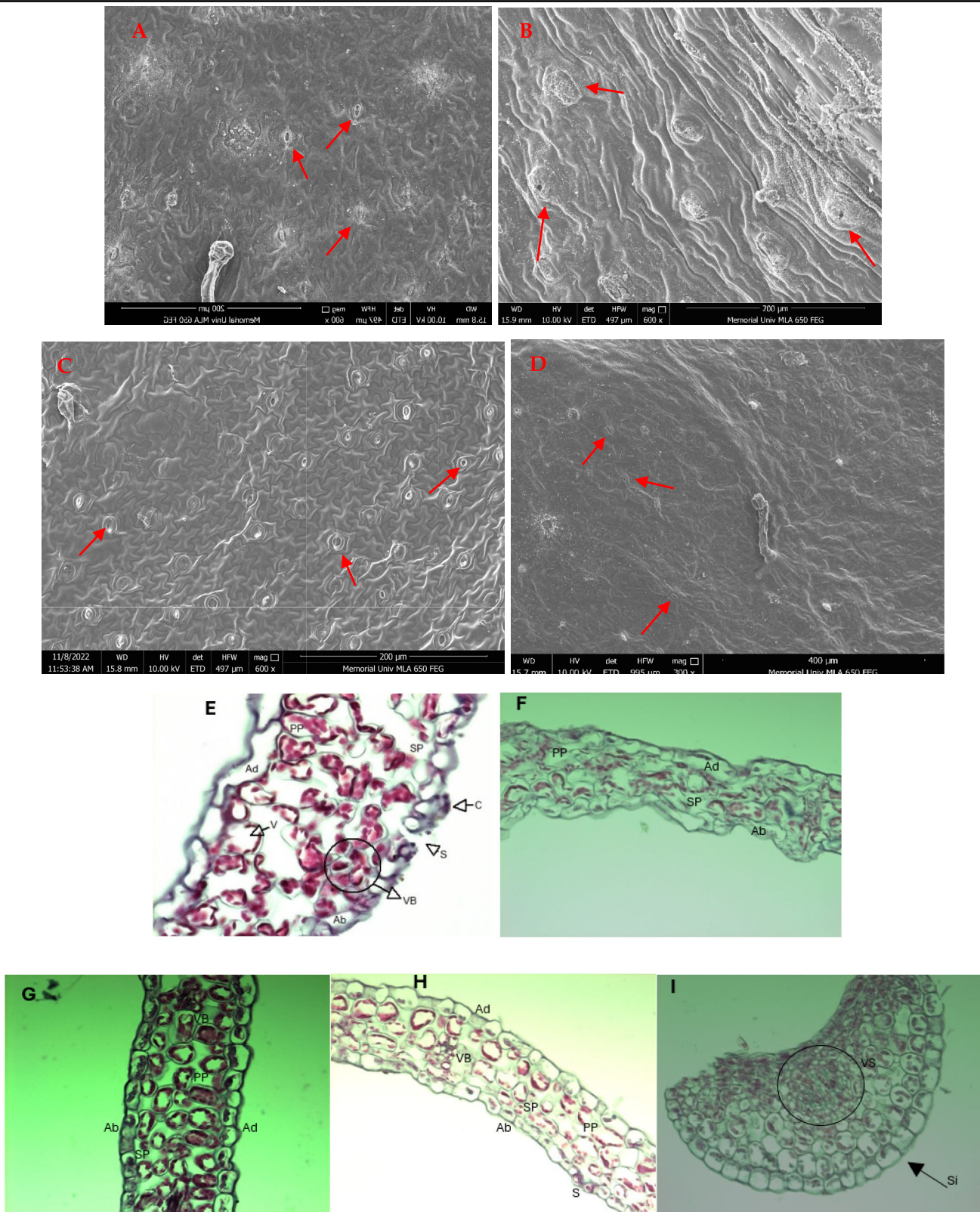


Fig. 5 Surface view of stomatal appearance and distribution in in vitro plants (ABC), *ex vitro* plants (D), and cross-sectional details of 4-week-old in vitro cultured leaves.; Ad: Adaxial surface, Ab: Abaxial surface, PP: Palisade parenchyma, SP: Spongy parenchyma, VB: Vascular bundle, S: Stomata, C: Cuticle, V: Vacuole, Si: Initiation of shoot organogenesis, VS: Vascular system; **A** Scanning electron microscopy showed less number of epidermal cell in VM31C leaf at semi solid condition; **B** Leaf from Growtek culture showed more disoriented stomata and irregular shaped epidermal cell; **C** RITA grown leaf present

highest number of stomata and organized elongated epidermal cell; **D** greenhouse-grown leaf shows less number irregular stomata, **E** GH (Greenhouse) grown leaf showed irregular shape of mesophyll tissue, **F** G (Growtek bioreactor) presented disoriented mesophyll tissue, **G** R (RITA bioreactor) exhibited organized mesophyll tissue, **H** ST (Semi solid tissue culture) showed less oriented mesophyll tissue, **I** O (TDZ 10 μM induced in vitro indirect organogenesis) showed vascular system in callus

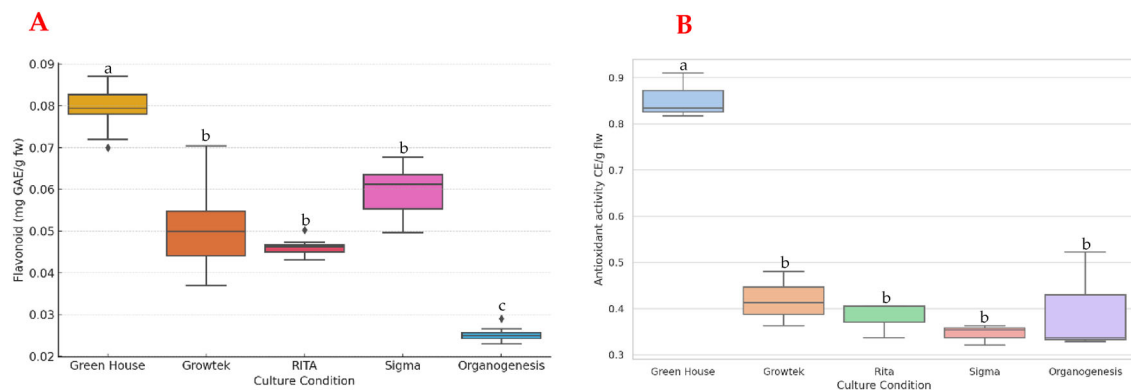


Fig. 6 Analysis of total flavonoid content and antioxidant activity. **A** One way ANOVA analysis of total flavonoid content; **B** One way ANOVA analysis of antioxidant activity. The Duncan multiple range test

followed data. Results are expressed as mean $\pm$ standard error (SE) for three replicates and indicate significant differences between treatments

Table 2 Statistical analysis of the effect of propagation methods and naturally growing greenhouse conditions on secondary metabolite production and antioxidant enzyme activities in [species]

Culture Conditions	Catalase ($\text{U g}^{-1} \text{ min}^{-1} \text{ fw}$)	Superoxide Dismutase ($\text{U g}^{-1} \text{ min}^{-1} \text{ fw}$)	Glutathione Reductase ($\text{nmoles NADPH min}^{-1} \text{ g}^{-1} \text{ fw}$)	Proanthocyanin (mg g^{-1})	Total Phenolics ($\text{mg GAE g}^{-1} \text{ fw}$)	Anthocyanin ($\text{mg g}^{-1} \text{ fw}$)
Green House	1.0 ± 0.2^b	0.19 ± 1.01^b	0.3 ± 0.04^b	8.3 ± 3.1^a	0.8 ± 0.02^a	26.0 ± 0.5^a
Leaf culture	2.6 ± 0.3^a	0.80 ± 1.2^a	0.1 ± 0.05^c	1.1 ± 0.01^b	0.05 ± 0.01^c	18.0 ± 0.7^b
Sigma	1.8 ± 0.1^b	0.32 ± 0.7^b	0.2 ± 0.04^b	3.6 ± 0.5^b	0.2 ± 0.02^b	20.0 ± 0.5^b
Growtek	2.8 ± 0.2^a	0.41 ± 0.8^b	0.2 ± 0.03^b	8.2 ± 2.0^a	0.8 ± 0.03^a	23.0 ± 0.4^a
RITA	1.6 ± 0.4^b	0.77 ± 1.1^b	0.4 ± 0.03^a	3.6 ± 0.9^b	0.6 ± 0.01^b	21.1 ± 0.7^b

Values represent means $\pm$ SE of three replicates. Tukey's test was performed to determine significant differences between treatment groups at $P \leq 0.05$. Different letters within a column indicate significant differences among the means. Different letters indicate statistically significant differences between treatments

Elucidation antioxidant enzymes, proanthocyanin, total phenolics and anthocyanin assay

Three different types of antioxidant enzymes were tested to analyze their enzyme activity. For CAT activity (Table 2), one way ANOVA was significant ($P \leq 0.05$) with their following descending order: Growtek culture ($2.8 \pm 0.2 \text{ U g}^{-1} \text{ min}^{-1} \text{ fw}$) > Leaf culture ($2.6 \pm 0.3 \text{ U g}^{-1} \text{ min}^{-1} \text{ fw}$) > Sigma culture ($1.8 \pm 0.1 \text{ U g}^{-1} \text{ min}^{-1} \text{ fw}$) > RITA culture ($1.6 \pm 0.4 \text{ U g}^{-1} \text{ min}^{-1} \text{ fw}$) > Greenhouse ($1.0 \pm 0.2 \text{ U g}^{-1} \text{ min}^{-1} \text{ fw}$). Growtek explants and leaf cultured shoot have almost the same level of catalase activation as like Sigma culture and RITA cultured explants.

Among all cultured plants, the leaf cultured shoot's leaf had the greatest activity of the SOD enzymes measured ($0.80 \pm 1.2 \text{ U g}^{-1} \text{ min}^{-1} \text{ fw}$) (Table 2). The descending order of this enzyme activity is: Leaf culture ($0.80 \pm 1.2 \text{ U g}^{-1} \text{ min}^{-1} \text{ fw}$) > RITA culture ($0.77 \pm 1.1 \text{ U g}^{-1} \text{ min}^{-1} \text{ fw}$) > Growtek culture ($0.41 \pm 0.8 \text{ U g}^{-1} \text{ min}^{-1} \text{ fw}$) > Sigma culture ($0.32 \pm 0.7 \text{ U g}^{-1} \text{ min}^{-1} \text{ fw}$) > Greenhouse ($0.19 \pm 1.01 \text{ U g}^{-1}$

$\text{min}^{-1} \text{ fw}$). Their SOD activity was significant in the one-way ANOVA ($P \leq 0.05$).

Comparing other cultured plants, the Growtek cultured plant's leaf has the greatest degree of GR enzyme activity ($7.04 \text{ nmoles NADPH min}^{-1} \text{ g}^{-1} \text{ fw}$) (Table 2). One-way ANOVA was used for the test, and $P \leq 0.05$ was used for statistical significance. For GR, the descending order is this following: RITA culture ($0.4 \pm 0.03 \text{ nmoles NADPH min}^{-1} \text{ g}^{-1} \text{ fw}$) > Greenhouse ($0.3 \pm 0.04 \text{ nmoles NADPH min}^{-1} \text{ g}^{-1} \text{ fw}$) > Sigma culture ($0.2 \pm 0.04 \text{ nmoles NADPH min}^{-1} \text{ g}^{-1} \text{ fw}$) > Growtek culture ($0.2 \pm 0.03 \text{ nmoles NADPH min}^{-1} \text{ g}^{-1} \text{ fw}$) > Leaf culture ($0.1 \pm 0.05 \text{ nmoles NADPH min}^{-1} \text{ g}^{-1} \text{ fw}$). Less variation exists between the samples.

Similarly, as total flavonoid and antioxidant activity; proanthocyanin has the same sequencing order with a statistically significant p value ($P \leq 0.05$). For proanthocyanin, the decreasing order among different cultured plants is Green House > Growtek culture > RITA culture > Sigma culture > Leaf culture (Table 2). These findings show that plants grown

in greenhouses and cultivated in Growtek have nearly identical results.

Like total flavonoid, antioxidant activity, and proanthocyanin assay, anthocyanin also depicts the same way to produce anthocyanin content (Table 2). For anthocyanin assay, the sequencing order is: Green House ($26.0 \pm 0.5 \text{ mg g}^{-1}$) > Growtek culture ($23.0 \pm 0.4 \text{ mg g}^{-1}$) > RITA culture ($21.1 \pm 0.7 \text{ mg g}^{-1}$) > Sigma culture ($20.0 \pm 0.5 \text{ mg g}^{-1}$) > Leaf culture ($18.0 \pm 0.7 \text{ mg g}^{-1}$). The test was done with statistically significant ($P \leq 0.05$).

The same as CAT activity, the highest point ($0.8 \pm 0.03 \text{ mg GAE g}^{-1} \text{ flw}$) was occupied by growtek cultured plants (Table 2), while the least was occupied by leaf cultured shoot ($0.05 \pm 0.01 \text{ mg GAE g}^{-1} \text{ flw}$). For total phenolics: Growtek culture ($0.8 \pm 0.03 \text{ mg GAE g}^{-1} \text{ flw}$) > Green House ($0.8 \pm 0.02 \text{ mg GAE g}^{-1} \text{ flw}$) > RITA culture ($0.6 \pm 0.01 \text{ mg GAE g}^{-1} \text{ flw}$) > Sigma culture ($0.2 \pm 0.02 \text{ mg GAE g}^{-1} \text{ flw}$) > Leaf culture ($0.05 \pm 0.01 \text{ mg GAE g}^{-1} \text{ flw}$). Growtek cultured plants and Greenhouse grown plants had similar results (Table 2).

Correlation among secondary metabolites

The CAT activity determined by H_2O_2 has a good linear regression ($r^2 = 0.396$) with GR. The total phenolics of leaf extracts of 3 different cultured explants, one leaf culture-derived plant, and one greenhouse-grown plant displayed a significant linear connection ($r^2 = 0.189$) with total flavonoid contents assessed by catechin components. The regression between total phenolics to antioxidant activity ($r^2 = 0.139$), proanthocyanidin ($r^2 = 0.229$), and anthocyanin ($r^2 = 0.169$) has a good linear connection separately. Compared to antioxidant capacity and total flavonoid content ($r^2 = 0.526$), the correlation coefficient between anthocyanin and total flavonoid content was higher at $r^2 = 0.645$. Antioxidant and anthocyanin had a favorable connection ($r^2 = 0.443$ and $r^2 = 0.683$, respectively) (Fig. 7).

Discussion

This study explores the efficacy of liquid bioreactor systems (Growtek and RITA) for micropropagation of black huckleberry for the first time. This protocol provides a promising alternative to traditional semi-solid media used for propagation of black huckleberry (Debnath and Barney 2012). The results demonstrate that Growtek bioreactor system significantly enhance shoot proliferation, yielding 76% regeneration, compared to RITA system (40.3%) Table 1. These findings align with earlier studies on other blueberry, cranberry, lingonberry, raspberry (Arigundam et al. 2020; Debnath 2009b, 2014, 2017; Debnath and Arigundam 2020),

which also reported superior growth in liquid media due to improved nutrient absorption and aeration.

The study highlights the importance of CK hormones with their concentrations in shoot regeneration and proliferation during indirect organogenesis. Different concentrations of CK were used in black huckleberries (Debnath and Barney 2012) for callus formation. In the current study, two different CK hormones: TDZ ($10 \mu\text{M}$) and ZEA ($8.5 \mu\text{M}$) were found effective in inducing indirect organogenesis for plant regeneration from leaf explant. TDZ is classified as a CK due to its cytokinin-like effects, in in vitro cultures, it exhibits both auxin and cytokinin like activities across various plant species (Ghosh et al. 2018). The effect of TDZ underscores its suitability for huckleberry micropropagation, though further optimization may be necessary to balance shoot proliferation with root development (Fritsche et al. 2022). When huckleberry leaves were inoculated on a BM supplemented with $10 \mu\text{M}$ TDZ, development of premordium was visible after 5 weeks (Fig. 1B). These formed globular like structures 8 weeks (Fig. 4A). Other CK hormones; ZEA is a naturally occurring CK classified under isoprenoid cytokinins. may be the most effective in promoting a high number of shoots and supporting sustained growth in *Vaccinium* species (Debnath et al. 2023; Sharma et al. 2023; Sikdar et al. 2021). $8.5 \mu\text{M}$ ZEA was also significant for inducing callus formation to generate vigorous shoots. ZEA is thought to be synthesized from isopentenyladenine through the hydroxylation of a terminal methyl group on the side chain (Sun et al. 2023). On BM (Debnath and McRae 2001) containing Gibberellic acid and 6-Benzylaminopurine hormones, indirect organogenesis from leaf culture were excluded from the study due to less plant vigour. The plant geranium, where exogenous Gibberellic acid treatment on MS media prevented the development and expression of many somatic embryo stages (Hutchinson et al. 1997). For further shoot proliferation, $4 \mu\text{M}$ ZEA were used in BM (Debnath and Barney 2012; Debnath and McRae 2001). In our study, compared to semi-solid culture, liquid cultured bioreactor (Growtek) system got highest shoot regeneration (76 ± 0.57), leaves per shoot (10.7 ± 0.15) and shoot vigour (7.33 ± 0.66).

Hyperhydricity, a common challenge in liquid culture systems, was noted predominantly in bioreactor, resulting in irregular stomatal and mesophyll structures (Gao et al. 2018; Louro et al. 1999; Picoli et al. 2008a, b; Wafa et al. 2016). Such abnormalities have been previously few reported in hyperhydric plants like blueberries and other *Vaccinium* plants (Arigundam et al. 2020; Gao et al. 2018; Molnar et al. 2024), indicating a universal stress response to prolonged immersion, but not in clear in huckleberry. The leaf stomata from RITA cultured leaf (Fig. 5C) exhibited precisely ordered trichomes and numerous growths of stomata with a number of epidermal cell compared to semi solid-cultured leaf (Fig. 4A) and greenhouse-produced leaf (Fig. 5D). RITA grown leaf

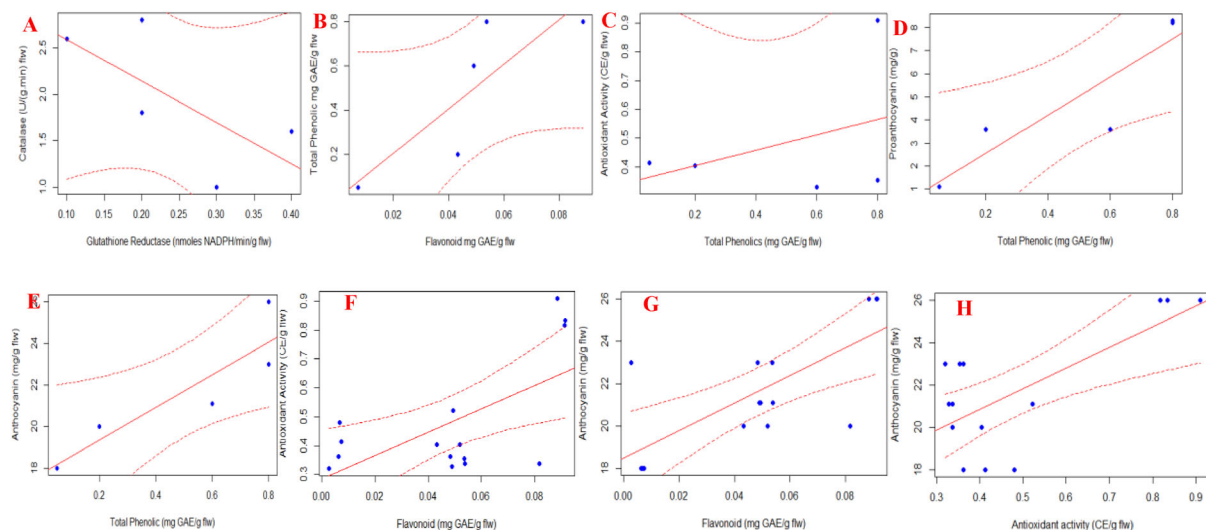


Fig. 7 Linear regression using quantile plot among secondary metabolites in greenhouse, different environment's tissue culture explants and leaf cultured shoot. Data were investigated based on means $\pm$ SD, $n = 3$. The associations were statistically significant with a confidence level of 95% ($\alpha = 0.05$); **A** CAT activity ($\text{U g}^{-1} \text{min}^{-1} \text{flw}$) and GR ($\text{nmol NADPH min}^{-1} \text{g}^{-1} \text{flw}$); **B** total phenol activity ($\text{mg GAE g}^{-1} \text{flw}$) and total flavonoid content ($\text{CE g}^{-1} \text{flw}$); **C** total phenol activity ($\text{mg GAE g}^{-1} \text{flw}$) and antioxidant activity ($\text{mg GAE g}^{-1} \text{flw}$); **D** total phenol activity ($\text{mg GAE g}^{-1} \text{flw}$) and proanthocyanin content (mg g^{-1}); **E** total phenol activity ($\text{mg GAE g}^{-1} \text{flw}$) and anthocyanin content (mg g^{-1}); **F** total flavonoids ($\text{CE g}^{-1} \text{flw}$) and antioxidant activity ($\text{mg GAE g}^{-1} \text{flw}$); **G** total flavonoids ($\text{CE g}^{-1} \text{flw}$) and anthocyanin content (mg g^{-1}); **H** antioxidant activity ($\text{mg GAE g}^{-1} \text{flw}$) and anthocyanin content (mg g^{-1})

has a higher density of stomata than a greenhouse-grown leaf (Fig. 5C). However, hyperhydric blueberry plantlets were distinguished by malformed stomata, reduced stomatal aperture, disoriented epidermal cell, disintegrated membrane structures (Fig. 5B). Hyperhydric plants also had severely collapsed vascular tissues (Jausoro et al. 2010; Louro et al. 1999). Furthermore, hyperhydrated blueberry plantlets' vacuole volume and the number of mitochondria per cell were noticeably increased (Gao et al. 2018). The liquid bioreactor (Growtek) cultivated plants, noting closed and thick stomata on a scanning electron microscope (Fig. 5B), which indicates to become a hyperhydric plant. The histological observation revealed several physical changes in different cultured plants. Compared to leaves grown in greenhouse (Fig. 5E), RITA (Fig. 5G), and semi solid (Fig. 5H) conditions, leaves grown in Growtek (Fig. 5F) exhibited altered and degenerated vacuoles. Tannins were initially abnormally stacked and later evenly distributed within the vacuoles (Reig-Armiñana et al. 2004) in hyperhydric plants. Under Growtek (Fig. 5F) conditions, mesophyll cells collapsed and compressed. Reig-Armiñana et al. (2004) reported that the cytoplasmic volume of the cells decreased, and the stomata became nonfunctional. In semi solid (Fig. 5H) conditions, the vascular bundle was absent, and both palisade and spongy parenchyma were thick with enlarged intercellular spaces. In callus formation, our finding indicates that TDZ induced leaf (Fig. 5I) initiate organogenesis through meristematic center by interacting with exogenous plant growth regulator: TDZ.

$\text{g}^{-1} \text{flw}$) and antioxidant activity ($\text{mg GAE g}^{-1} \text{flw}$); **D** total phenol activity ($\text{mg GAE g}^{-1} \text{flw}$) and proanthocyanin content (mg g^{-1}); **E** total phenol activity ($\text{mg GAE g}^{-1} \text{flw}$) and anthocyanin content (mg g^{-1}); **F** total flavonoids ($\text{CE g}^{-1} \text{flw}$) and antioxidant activity ($\text{mg GAE g}^{-1} \text{flw}$); **G** total flavonoids ($\text{CE g}^{-1} \text{flw}$) and anthocyanin content (mg g^{-1}); **H** antioxidant activity ($\text{mg GAE g}^{-1} \text{flw}$) and anthocyanin content (mg g^{-1})

Biochemical analysis revealed that greenhouse cultured plants had the highest levels of anthocyanins, total phenolics, and antioxidant activities, confirming the superior bioactive profile in *ex vitro* conditions. Interestingly, Growtek cultured plants exhibited elevated phenolic content and CAT activity, suggesting that controlled in vitro conditions can mimic some aspects of natural growth condition. This reinforces the potential of bioreactor systems for producing high-value black huckleberry while maintaining bioactive properties. It was previously studied that *Vaccinium* berries have considerable flavonoid content. A tricyclic $\text{C}_6\text{C}_3\text{C}_6$ "flavonoid skeleton" makes up the standard molecular structure of all the polyphenolic chemicals in this widely studied category. Red huckleberries came with $0.16 \text{ CE g}^{-1} \text{flw}$, whilst the black huckleberry having the highest catechin content ($0.24 \text{ CE g}^{-1} \text{flw}$) (Taruscio et al. 2004). According to our findings, black huckleberry plants cultivated in greenhouses had the most significant total flavonoid activity at $0.09 \text{ CE g}^{-1} \text{flw}$ (Fig. 6A). The red huckleberry has no measurable myricetin ($0.005 \text{ CE g}^{-1} \text{flw}$) and the least quantity of quercetin ($0.0093 \text{ CE g}^{-1} \text{flw}$) (Moyer et al. 2002; Taruscio et al. 2004). Determining antioxidant activity is complicated since it involves the positive and negative impacts of plants in many different environmental conditions. Although the exact causes of the more incredible antioxidant content in plants cultivated in greenhouses are unclear, age and culture conditions of the plants may play a role (Ghosh et al. 2018). In the presence of antioxidant molecules, the stable free radical DPPH

is reduced to an uncolored solution when it is dissolved in ethanol (Ghosh et al. 2018). According to measurements, the evergreen huckleberry had the highest total antioxidant capacity ($41.1 \mu\text{mol}$ of TE g^{-1} of FW) (Trolox Equivalent) while the red huckleberry had the lowest ($7.3 \mu\text{mol}$ of TE g^{-1} of FW). The black huckleberry produced an outstanding quantity ($0.86 \text{ mg GAE g}^{-1}$ flw) using the universal DPPH activity test in greenhouse-grown plants (Fig. 6B). However, the bulk of the antioxidants (65–86% of their polyphenolic profile) were present in the cascade, black, and evergreen huckleberries and the oval-leaf blueberry (Taruscio et al. 2004).

Plants often contain anthocyanins and flavonols as glycosides, while these glycosides can also be found in acetylated forms. For example, the anthocyanidin can exist in a variety of glycosidic forms, including acetylated cyanidin-3-glucose and the glycosidic forms cyanidin-3-galactoside, cyanidin-3-glucoside, and cyanidin-3-arabinoside (Debnath et al. 2023; Kalt et al. 1999). Due to its darker pigmentation, the red huckleberry had the lowest amounts of cyanidin (0.11 mg of $\text{C}_3\text{G g}^{-1}$) (cyanidin 3-glucoside). The most amazing antiradical activity would be displayed by the evergreen huckleberry and oval leaf blueberry, which had the greatest total anthocyanin content (1.69 mg of $\text{C}_3\text{G g}^{-1}$). But according to our research, greenhouse grown plants produced the most anthocyanin ($26.0 \pm 0.5 \text{ mg g}^{-1}$ flw) (Table 2). On the other hand, according to Taruscio study (Taruscio et al. 2004), the red huckleberry had the lowest amounts of phenolics, anthocyanins, and antiradical capability.

The growth hormones utilized in in vitro system may up or down-regulate the genes involved in the biosynthesis pathway, which is crucial for producing plant phenolic content (Ghosh et al. 2018). The majority of phenolic compounds are known to have antibacterial effects. The evergreen huckleberry and oval-leaf blueberry had the greatest amounts of TPH (2.84 and $2.81 \text{ mg GAE g}^{-1}$, respectively), while the red huckleberry had the lowest level of total phenolic content ($0.81 \text{ mg GAE g}^{-1}$ FW). The maximum quantity ($0.8 \pm 0.03 \text{ mg GAE g}^{-1}$ flw) was obtained at bioreactor (Growtek) cultivated plants than other cultured plants. The total phenolics were reported $1.70 \text{ mg GAE g}^{-1}$ in frozen berries (Taruscio et al. 2004).

This study provides the first estimates of proanthocyanin content and the activity of three antioxidant enzymes (CAT, SD, and GR) in huckleberry plants. However, CAT and SOD activities in the skin of blueberry fruit increased by 320 and $400 \text{ U g}^{-1} \text{ min}^{-1}$, respectively (Sun et al. 2018). In Growtek grown and leaf cultured regenerated shoot, the greatest CAT activity and SOD activity showed $2.8 \pm 0.2 \text{ U g}^{-1} \text{ min}^{-1}$ and $0.80 \pm 1.2 \text{ U g}^{-1} \text{ min}^{-1}$ flw (Table 2), respectively. GR is an endogenous antioxidant with significant antioxidant properties that functions as a co-factor of GPx in the cell's redox state. Despite the body's built-in antioxidant defense system,

oxidative stress can happen for various causes, including mutations or improper ROS activation (Wang et al. 2017). Bioreactor (RITA) grown plants also had the highest amount of GR ($0.4 \pm 0.03 \text{ nmoles NADPH min}^{-1} \text{ g}^{-1}$ flw) (Table 2). The maximum proanthocyanin concentration in greenhouse-grown plants was $8.3 \pm 3.1 \text{ mg g}^{-1}$ flw (Fig. 7D). According to Goyali (Goyali et al. 2015), Blueberry wild clone QB 9C scored the highest at 2.5 CE mg g^{-1} flw.

Conclusion

This study establishes a novel protocol for the first time for the micropropagation of black huckleberry. This liquid bioreactor system demonstrates clear advantages over semi-solid media. The findings provide a foundation for large-scale commercial propagation and further research into the genetic and epigenetic stability of regenerated plants. Additionally, the study opens avenues for exploring the biochemical pathways underlying phenolic and antioxidant compound biosynthesis, enhancing the crop's medicinal value.

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Author contributions RB: performed and organized the research and wrote the original draft. SK: assisted in the biochemical analysis and statistical data analysis. SM: assists in writing manuscripts and Morphological data analysis. AUI and HDM: served as a cosupervisor/ supervisory committee member and reviewed and edited the manuscript. SCD: originated the concept, supervised the research, and interpreted the results, and played a role in funding acquisition, manuscript writing, reviewing and editing. All authors approved the final version of the manuscript.

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Data availability No datasets were generated or analysed during the current study.

Declarations

Competing interests The authors confirm that they have no financial or personal conflicts of interest that could have influenced the findings presented.

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