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In Vitro Shoot Induction of Highbush Blueberry (*Vaccinium corymbosum* L.)

Roshani G Jagtap * and Rohini M Kolekar
Vidya Pratishthan's College of Agricultural Biotechnology, Baramati-413 133
Affiliated to Mahatma Phule Krishi Vidyapeeth, Rahuri, Ahmednagar (Maharashtra)

ABSTRACT

The highbush blueberry (*Vaccinium corymbosum* L.) fruits has experienced a substantial rise, largely due to their nutritional and medicinal qualities. Propagation through *in vitro* culture has been considered the most effective method for a rapid increase of disease-free propagules on a year-round basis. This study was focused on the establishment of a high-efficiency *in vitro* tissue culture and rapid propagation technology system by using blueberry leaves and nodal segment. The experiment included a comparative analysis of WPM (Woody Plant Medium) with different growth hormones. For callus induction, leaf explants were cultured on WPM enriched with different combinations of BAP (0.0, 0.5 and 1.0 mg/l) and/or 2,4-D (1.0, 1,5 and 2,0 mg/l) , as well as WPM with Zeatin (ZT) (0.1 to 0.5 mg/l.) alone. The comparative analysis for callus induction was ranged from 50 to 100 per cent. The formulation of WPM with 0.5 mg/L BAP and 1 mg/L 2,4-D achieved a full callus formation (100 %). For shoot induction, these Calli were transferred to a shoot induction medium provided with BAP (0.0, 0.5 and 1.0 mg/l) and/or 2,4-D (1.0, 1,5 and 2,0 mg/l) , as well as WPM with Zeatin (ZT) (0.1 to 0.5 mg/l) separately. The nodal explants were also used for the shoot induction by culturing them on WPM provided with ZT (0.1 to 0.5 mg/l). Successful shoot formation **(**93.3 %) was observed on WPM with ZT at 0.5 mg/l.

Keywords: Highbush Blueberry, *Vaccinium corymbosum, In vitro,* Woody Plant Medium etc.

INTRODUCTION

The highbush blueberry (*Vaccinium corymbosum* L.) is a member of the Ericaceae family (Monika *et al*, 2022). As a tetraploid, rhizomatous shrub that undergoes cross-pollination, the blueberry holds considerable commercial value on a global scale, with Chile being a prominent nation in both production and exportation (Ariel *et al*, 2013). Blueberries are divided into five major categories: 1) northern highbush (*Vaccinium corymbosum L.*); 2) southern highbush, which arises from the hybridization of *V. corymbosum*, *Vaccinium darrowii Camp*, and other blueberry species; 3) lowbush, which comprises *Vaccinium angustifolium Ait, Vaccinium myrtilloides Michx, and Vaccinium boreale Hall* and *Aald*.; 4) half-high, a hybrid resulting from the crossing of highbush and lowbush types; and 5) rabbiteye (*Vaccinium virgatum Ait*.) (Dongliang *et al*, 2018). Based on information from the International Blueberry Organization (IBO), the total area registered for blueberry production in 2023 reached 267,000 ha.

Numerous research investigations have demonstrated that blueberries possess therapeutic potential for a variety of health conditions, including diabetes management, the prevention of diabetic retinopathy, neuroprotection, the treatment of malignant tumours, chronic disease mitigation, cardiovascular disease prevention, antimicrobial properties, enhancement of memory, and protection against Alzheimer's disease (Abou El-Dis *et al*, 2021). Traditionally, blueberry propagation has been carried out using cuttings from coniferous, semi-coniferous, and deciduous species, as well as rhizomatous cuttings from specific clones. However, this method presents several challenges, including a notably low success rate for rooting in many genotypes, extended timeframes for propagating and bringing newly developed plants to market, and various phytosanitary issues (Monika *et al*, 2022). Traditional techniques for propagation are often marked by their slow progress, intensive labour demands, and a pronounced dependence on environmental conditions and seasonal cycles (Correia *et al*, 2024). Micropropagation is an extremely efficient technique for propagating new blueberry cultivars, enabling the quick production of many plants. As a result, the practice of micro propagating blueberries has significantly increased over the last thirty years (Testsumura *et al*, 2012). Techniques of tissue culture propagation serve as an effective method to produce virus-free plants (Monika *et al*, 2022). The propagation

Corresponding Author's Email - roshanijagatap1553@gmail.com

Table 1. Combinations of growth hormones (BAP and 2,4-D) for callus induction from the leaves of Blueberry.

Sr. No.	Treatment	Treatment combination
1	T_1	Control
2	T_2	WPM + 0.0 mg/L BAP + 1.0 mg/L 2,4-D
3	T_3	WPM + 0.5 mg/L BAP + 1.0 mg/L 2,4-D
4	T_4	WPM + 1.0 mg/L BAP + 1.0 mg/L 2,4-D
5	T_5	WPM + 0.0 mg/L BAP + 1.5 mg/L 2,4-D
6	T_6	WPM + 0.5 mg/L BAP + 1.5 mg/L 2,4-D
7	T_7	WPM + 1.0 mg/L BAP + 1.5 mg/L 2,4-D
8	T_8	WPM + 0.0 mg/L BAP + 2.0 mg/L 2,4-D
9	T_9	WPM + 0.5 mg/L BAP + 2.0 mg/L 2,4-D
10	T_{10}	WPM + 0.5 mg/L BAP + 2.0 mg/L 2,4-D

Table 2. Concentrations of zeatin for callus induction and shoot initiation of blueberry

Sr. No.	Treatment	Concentrations
1	T_1	WPM + 0.1 mg/L Zeatin
2	T_2	WPM + 0.2 mg/L Zeatin
3	T_3	WPM + 0.3 mg/L Zeatin
4	T_4	WPM + 0.4 mg/L Zeatin
5	T_5	WPM + 0.5 mg/L Zeatin

of blueberries through tissue culture is associated with a more extensive plant spread, leading to an increased bearing area and, as a result, higher yields compared to those obtained from conventional propagation methods utilizing softwood, single-node cuttings (Djurdjina *et al*, 2012)

.MATERIALS AND METHODS

Plant Material and Sterilization

For shoot regeneration, nodal segment as well as leaf Explant material was used. The nodal segments were taken from one year old disease-free highbush blueberry plant. The segments were thoroughly rinsed with running tap water for 15-20 min. and then soaked in a solution of 2-3 drops of Tween 20 diluted in sterile distilled water for 15 minutes. After this, the segments were washed with sterile distilled water. Under a laminar flow hood, nodal segments were treated with a 0.1% $HgCl_2$ solution for 5 minutes, followed by three rinses with sterile distilled water. The nodal segments were then exposed to 70% ethanol for 30 seconds, after which they were washed again with sterile distilled water. Explants measuring between 1 and 1.5 cm in length were excised from single node of *V. corymbosum* and subsequently transferred to a culture medium.

For shoot regeneration from the leaf explant, young, actively growing leaves were harvested from highbush blueberry plants. Initially, the leaves were thoroughly rinsed under running tap water. Subsequently, leaves were immersed in a solution of 2-3 drops of Tween 20 mixed with sterile distilled water for a duration of 5 minutes, followed by a wash with sterile distilled water. The leaves were then exposed to a 0.1% solution of $HgCl_2$ for 2 minutes and rinsed three times with sterile distilled water. Finally, the leaves were treated with 70% ethanol for 10 seconds and washed again with sterile distilled water before applying a fungicide treatment (M45/Bavistin).

Media Preparation

The basal culture medium designated for shoot induction is the WPM (McCown Woody Plant Medium), as referenced by Lloyd and McCown (1980). This medium is supplemented with 30g/l sucrose, 10g/l agar and pH maintained at 4.8 along with different concentrations of the growth hormones like zeatin, BAP and 2, 4-D for callus induction and shoot initiation (Table No. 1, 2).

Establishment and maintenance of Cultures

The establishment of callus culture induced from leaves

Sterilized leaves subjected to 2 to 3 diagonal cuts that were perpendicular to the main vein. The upper side of the leaves was positioned facing upward

Table 3. Percentage of callus induced from leaf explant supplemented with different concentration of BAP and 2,4-D after 30-45 days.

Treatment	Treatment combination	No. of. Explants inoculated	No. of explants responded	Per cent (%) Response
T_1	WPM medium (Cultured)	120	0	0.00
T_2	WPM+ 0.0mg/L BAP + 1.0mg/L 2,4-D	120	90	75.00
T_3	WPM+ 0.5mg/L BAP + 1.0mg/L 2,4-D	120	120	100.00
T_4	WPM+ 1.0mg/L BAP + 1.0mg/L 2,4-D	120	90	75.00
T_5	WPM+ 0.0mg/L BAP + 1.5mg/L 2,4-D	120	100	83.30
T_6	WPM+ 0.5mg/L BAP + 1.5mg/L 2,4-D	120	70	58.30
T_7	WPM+ 1.0mg/L BAP + 1.5mg/L 2,4-D	120	100	83.30
T_8	WPM+ 0.0mg/L BAP + 2.0mg/L 2,4-D	120	60	50.00
T_9	WPM+ 0.5mg/L BAP + 2.0mg/L 2,4-D	120	70	58.50
T_{10}	WPM+ 0.5mg/L BAP + 2.0mg/L 2,4-D	120	80	66.60

Table 4. Percentage of callus induced from leaf supplemented with different concentration of zeatin explant after 30-45 days.

Treatment	Treatment combination	No. of. Explants	No. of explants responded	Per cent (%) Response
T_1	WPM medium (Cultured)	40	7	17.50
T_2	WPM+ 0.1 mg/L Zeatin	40	23	57.50
T_3	WPM+ 0.2 mg/L Zeatin	40	35	87.50
T_4	WPM+ 0.3 mg/L Zeatin	40	20	50.00
T_5	WPM+ 0.4 mg/L Zeatin	40	30	75.00
T_6	WPM+ 0.5 mg/L Zeatin	40	20	50.00

Table 5. Percentage of shoot initiation from nodal explant supplemented with different concentration of zeatin explant after 45-50 days.

Treatment	Treatment combination	No. of. Explants	Avg. no. of shoots initiated	Per cent (%) Response
T_1	WPM medium (Cultured)	60	0	0
T_2	WPM+ 0.1 mg/L Zeatin	60	25	41.60
T_3	WPM+ 0.2 mg/L Zeatin	60	47	78.30
T_4	WPM+ 0.3 mg/L Zeatin	60	52	86.60
T_5	WPM+ 0.4 mg/L Zeatin	60	38	63.30
T_6	WPM+ 0.5 mg/L Zeatin	60	56	93.30

on the medium provided with different combinations of growth hormones (Table No. 1 and 2), while the lower side was ensured to be in full contact with the medium. The petri plates were then sealed and placed in a dark culture chamber maintained at 24°C for a period ranging from 28 to 30 d. Following this incubation, the dishes were removed and incubated at 24°C under a 16-hour light and 8-hour dark cycle for another 14 to 28 d. Cultures were maintained in the replicated manner.

Initiation of shoots through callus derived from leaves

After complete callus development, the Calli were transferred to a shoot induction medium provided with BAP (0.0, 0.5 and 1.0 mg/l) and/or 2,4-D (1.0, 1,5 and 2,0 mg/l) , as well as WPM with Zeatin (ZT) (0.1 to 0.5 mg/l) separately. These cultures were maintained in a growth chamber at a regulated temperature of 24±1 °C, with a light exposure duration of 16 hours. After

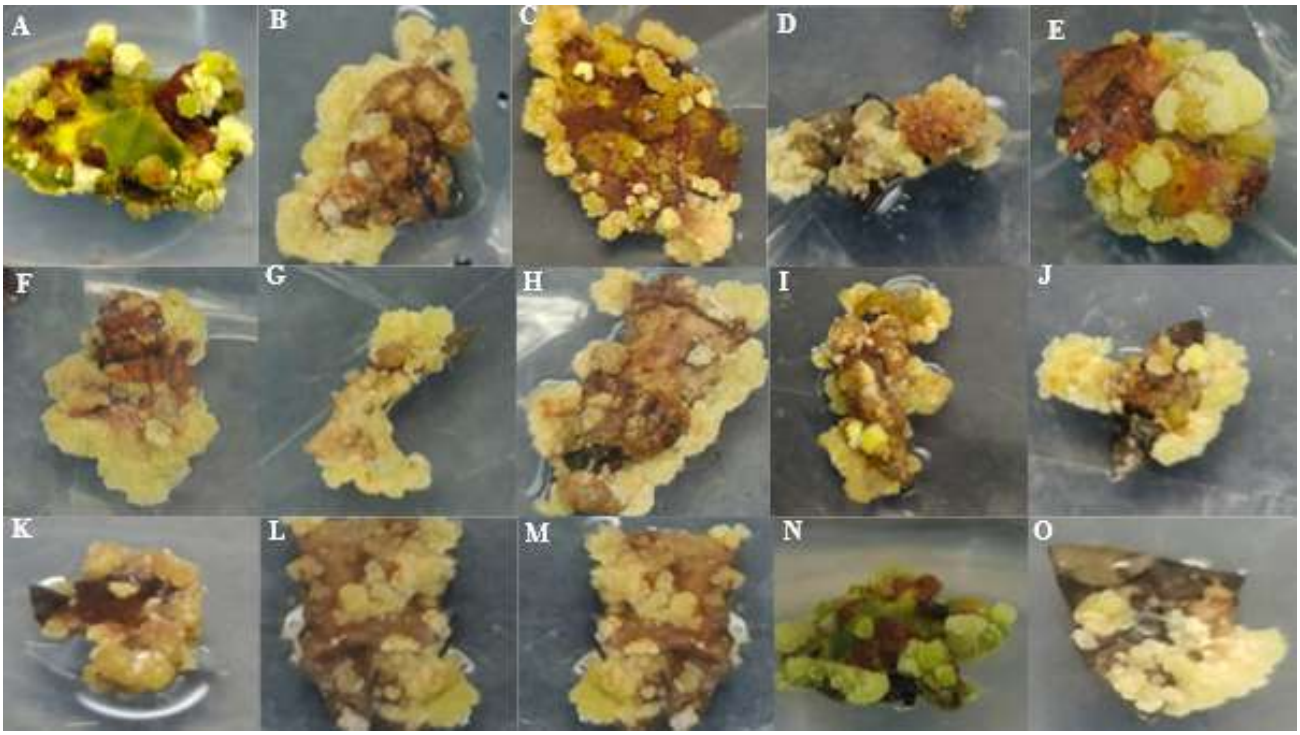

Plate No.1 Callus induced from leaf explants at different concentrations of PGRs (A) 0.1 mg/L Zeatin **(B)** 0.2 mg/L Zeatin **(C)** 0.3 mg/L Zeatin **(D)** 0.4 mg/L Zeatin **(E)** 0.5 mg/L Zeatin **(F)** 0.0 mg/L BAP + 1.0 mg/L 2,4-D **(G)** Control (**H)** 0.5 mg/L BAP + 1.0 mg/L 2,4-D **(I)** 1.0 mg/L BAP + 1.0 mg/L 2,4-D **(J)** 0.0 mg/L BAP + 1.5 mg/L 2,4-D **(K)** 0.5 mg/L BAP + 1.5mg/L 2,4-D **(L)** 1.0 mg/L BAP + 1.5 mg/L 2,4-D **(M)** 0.0 mg/L BAP + 2.0 mg/L 2,4-D **(N)** 0.5 mg/L BAP + 2.0 mg/L 2,4-D and **(O)** 0.5 mg/L BAP + 2.0 mg/L 2,4-D.

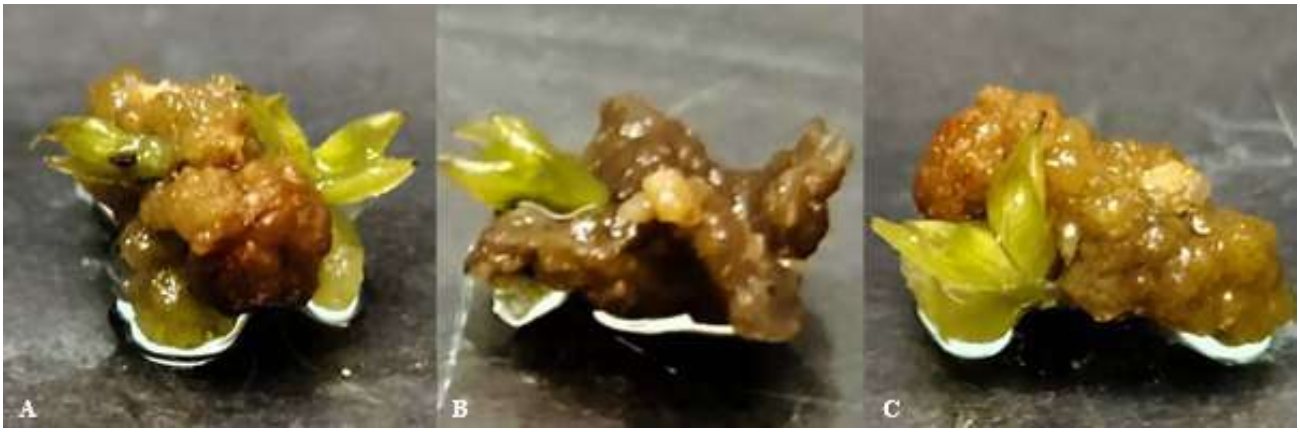

Plate No.2 Shoots initiated from callus at different concentration of Zeatin (A) 0.5 mg/L **(B)** 0.4 mg/L (**C)** 0.3 mg/L

15-20 days the shoot initiation through was observed on 0.5mg/l zeatin.

The establishment of shoots initials from nodal explant

The disinfected stem segments were aseptically segmented into single-nodal sections and cultured to initiate shoot development on WPM medium containing (0.1, 0.2, 0.3, 0.4, and 0.5 mg/l) zeatin with replications. After the inoculation, the culture bottles were incubated in a growth room at a controlled temperature of 24±1 °C, under a 16-hour light exposure.

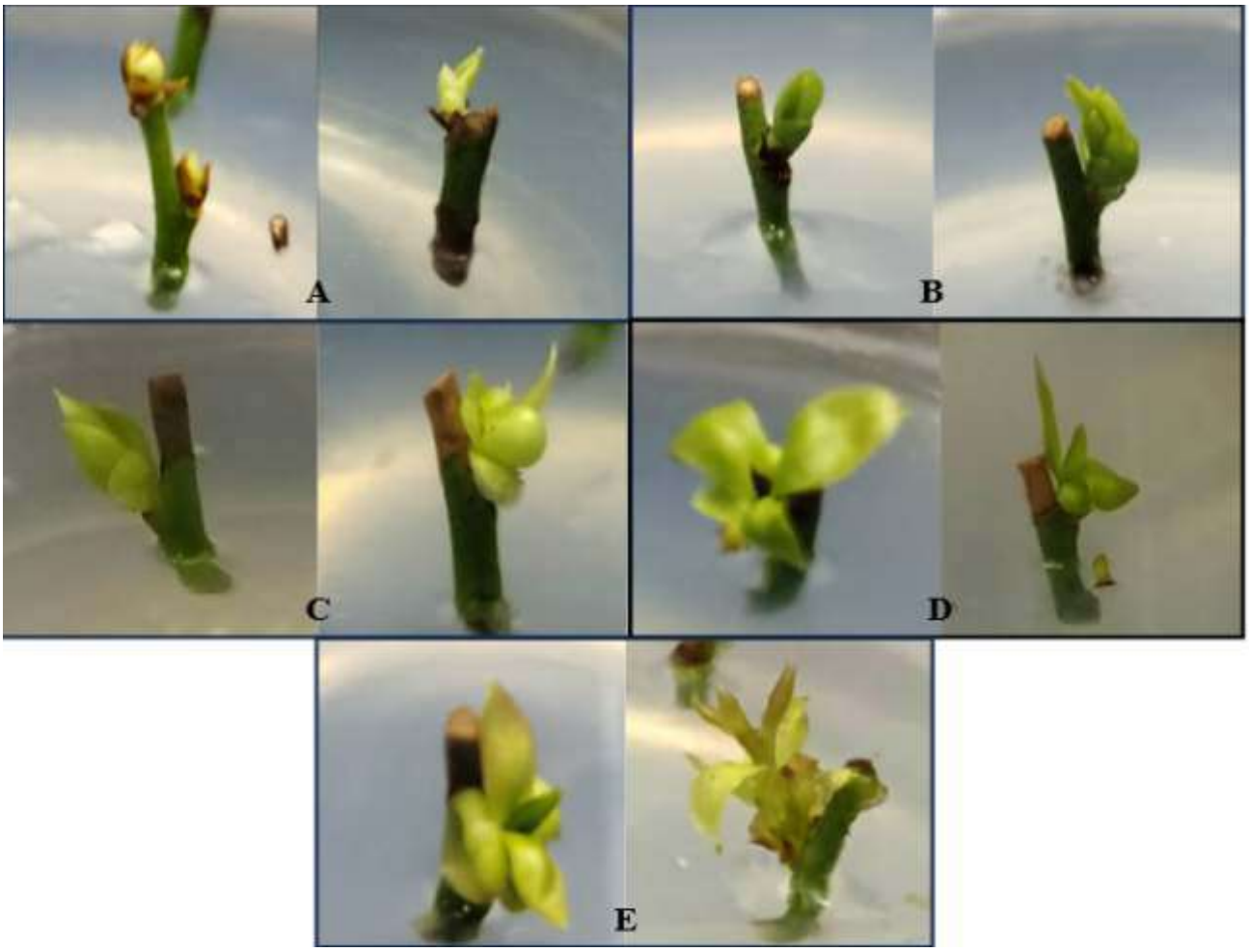

Plate No. 3. Shoot initiation from nodal explants at different concentration of Zeatin
(A) 0.1 mg/L Zeatin **B)** 0.2 mg/L Zeatin **(C)** 0.3 mg/L Zeatin **(D)** 0.4 mg/L Zeatin **(E)** 0.5 mg/L Zeatin

RESULTS AND DISCUSSION

This investigation outlines a comprehensive protocol for initiating shoots from nodal segments and callus derived from leaves *in vitro*. The results indicated that highbush blueberry can be successfully micro propagated through plant tissue culture using nodal segments as well as leaves as an explant. The sterilization procedure applied to leaves and nodal segments demonstrated high efficacy, resulting in a minimal contamination rate. The duration of exposure to 70% ethanol and 0.1% $HgCl_2$ is critical for the viability of the explants. Prolonged exposure to these sterilizing agents may lead to browning and subsequent death of the explants.

The results indicated that a concentration of 0.2 mg/L zeatin led to an 87.5% callus formation rate (Table No. 4). Additionally, the use of Woody Plant Medium (WPM) supplemented with 0.5 mg/L BAP and 1 mg/L 2,4-D resulted in complete callus formation (100 %) (Table No. 3) (Plate No. 1). The subculture of callus was executed on a shoot initiation medium containing zeatin from 0.1 to 0.5 mg/L (Table No. 2). After a period of 15 to 20 days of subculturing, shoot initiation was observed at concentrations of 0.3, 0.4, and 0.5 mg/l of zeatin (Plate No. 2).

Single-node segments cultured on WPM supplemented with zeatin at different concentrations (0.1 to 0.5 mg/L) demonstrated node proliferation. Zeatin at 0.5 mg/L showed optimal shoot formation (93.3 %) after 45-50 days of incubation period in growth room (Table No. 5 and Plate No. 3). The Woody Plant Medium (WPM) has been recognized as the most effective medium for the induction of callus and the

initiation of shoots from explants of blueberry leaves and stems, respectively. Cytokinin's such as zeatin, BAP and 2,4-D have been shown to promote callus development. Young leaves were cultured on a basal medium enriched with various plant growth regulators (PGRs), specifically the combination of 2,4-D and BAP, alongside the sole application of zeatin. Callus formation was observed after a 30- 45 d incubation period for each hormone combination. In prior research, WPM medium has proven to be an effective tool for inducing shoots across a range of Vaccinium species. In prior research, WPM medium has proven to be an effective tool for inducing shoots across a range of Vaccinium species. (Debnath *et al*, 2020). Research conducted in the past has also focused on the similar combinations of BAP and 2,4-D (Abou El-Dis *et al,* 2021).

CONCLUSION

The role of plant tissue culture in the vegetative propagation of blueberries is of great importance. This study successfully formulated an efficient protocol for the rapid micropropagation of highbush blueberry. The research involved experiments aimed at establishing callus cultures from leaves and initiating shoots from both callus and nodal segments. The results of this study confirm the successful in vitro regeneration of highbush blueberry, *Vaccinium corymbosum* L. The regeneration protocol developed here provides a framework that could improve shoot propagation systems for various other species in the *Vaccinium* genus. Furthermore, the implications of this research go beyond propagation techniques, as the established protocol can be utilized for biotechnological applications. By enabling the production of high-quality plant material, this work supports the broader objectives of sustainable agriculture and the conservation of genetic resources within the *Vaccinium* species. This innovative approach not only holds promise for improving propagation efficiency but also serves as a valuable resource for generating certified vegetative material. Such advancements are particularly significant in the context of horticultural practices and the commercial production of highbush blueberries.

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