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Polyploidy Induction of Wild Diploid Blueberry *V. fuscatum*

Emily Walter [1], Paul M. Lyrene [2] and Ye Chu [1,3,*]

[1] Horticulture Department, The University of Georgia, Tifton Campus, 2356 Rainwater Road, Tifton, GA 31793, USA; emily.walter@uga.edu
[2] Horticultural Sciences Department, University of Florida, Gainesville, FL 32601, USA; lyrene@ufl.edu
[3] Institute of Plant Breeding, Genetics and Genomics, The Plant Center, The University of Georgia, Tifton Campus, 2356 Rainwater Road, Tifton, GA 31793, USA
* Correspondence: ychu@uga.edu

Abstract

Diploid *Vaccinium fuscatum* is a wild blueberry species with a low chilling requirement, an evergreen growth habit, and soil adaptability to southeast US growing regions. Regardless of its potential to improve the abiotic and biotic resilience of cultivated blueberries, this species has rarely been used for blueberry breeding. One hurdle is the ploidy barrier between diploid *V. fuscatum* and tetraploid cultivated highbush blueberries. To overcome the ploidy barrier, vegetative shoots micro-propagated from one genotype of *V. fuscatum*, selected because it grew vigorously in vitro and two southern highbush cultivars, 'Emerald' and 'Rebel,' were treated with colchicine. While shoot regeneration was severely repressed in 'Emerald' and 'Rebel,' shoot production from the *V. fuscatum* clone was not compromised at either 500 µM or 5000 µM colchicine concentrations. Due to the high number of shoots produced in vitro via the *V. fuscatum* clone shoots of this clone that had an enlarged stem diameter in vitro were subjected to flow cytometer analysis to screen for induced polyploidy. Sixteen synthetic tetraploid *V. fuscatum*, one synthetic octoploid 'Emerald,' and three synthetic octoploid 'Rebel' were identified. Growth rates of the polyploid-induced mutants were reduced compared to their respective wildtype controls. The leaf width and length of synthetic tetraploid *V. fuscatum* and synthetic octoploid 'Emerald' was increased compared to the wildtypes, whereas the leaf width and length of synthetic octoploid 'Rebel' were reduced compared to the wildtype controls. Significant increases in stem thickness and stomata guard cell length were found in the polyploidy-induced mutant lines compared to the wildtypes. In the meantime, stomata density was reduced in the mutant lines. These morphological changes may improve drought tolerance and photosynthesis in these mutant lines. Synthetic tetraploid *V. fuscatum* can be used for interspecific hybridization with highbush blueberries to expand the genetic base of cultivated blueberries.

Keywords: polyploidy induction; tissue culture; colchicine; flow cytometry; blueberries

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1. Introduction

Blueberries have many health benefits such as improving cognitive and digestive health, and preventing cardiovascular diseases, diabetes, and muscular injuries [1]. Public awareness of these health benefits led to a steady increase in blueberry consumption and market demand [2]. As one of the world-leading blueberry producers, the United States produced 333,660 tons of blueberries in 2023, which was three times more than the production in 2000 [3]. The US blueberry industry generated $4.7 billion of economic impact by sustaining over 44,000 full-time jobs each year [4]. It is predicted that the market

demand for blueberries will continue to rise in the coming years due to the year-round market availability of blueberries, convenience for consumption, and awareness of health benefits among consumers [5]. To meet the demand of the expansion of the blueberry industry, developing high-yielding blueberry cultivars that are not only adapted to the local growing environment but also have broad harvest windows is imperative.

Blueberries (*Vaccinium* sect. *Cyanococcus*) are native to North America [6]. Major types of cultivated blueberries include northern highbush and southern highbush (both interspecific hybrids based on *V. corymbosum*, $2n = 4x = 48$, NHB and SHB), lowbush (*V. augustifolium*, $2n = 4x = 48$, LB), and rabbiteye blueberries (*V. virgatum*, $2n = 6x = 72$, RE). *V.* sect. *Cyanococcus* was divided into 9 diploid, 12 tetraploid, and 3 hexaploid species based on morphological characteristics by Camp [6]. Vander Kloet [7] later lumped nine highbush species at diploid, tetraploid, and hexaploid levels into *V. corymbosum*. Efforts are underway to resolve the taxonomic dispute based on phylogenetic analyses and comprehensive sampling [8]. In this study, we adopted the Camp treatment since it is widely used in the relevant literature.

Interspecific hybridization among cultivated species and wild germplasm played a key role in blueberry cultivar development. The domestication and breeding of highbush blueberry started with interspecific hybridization between *V. corymbosum* and *V. angustifolium* in the early 1900s [9]. Backcrosses to wild highbush selections from New Jersey and intercrosses among selected progenies resulted in the release of northern highbush blueberry cultivars [10,11]. Subsequent interspecific hybridization enabled the expansion of blueberry cultivation to a wide range of soils and climate conditions [12]. This was exemplified by the development of SHB blueberries that thrive in regions with a warmer climate through introgression of low-chill and soil adaptability from *V. virgatum* and *V. darrowii* ($2n = 2x = 24$) into the NHB genetic background [13]. In addition to *V. virgatum* and *V. darrowii*, another diploid species, *V. elliottii* ($2n = 2x = 24$), contributed to the fruit flavor, fragrance, upland soil tolerance, and disease resistance of SHB cultivars such as 'Snowchaser', 'Carteret', and 'Kestrel' [14]. Signatures of introgression from both hexaploid and diploid wild blueberries were identified in SHB through population structure analysis [15].

Compared to the active breeding efforts with *V. virgatum*, *V. darrowii*, and *V. elliottii*, little attention has been paid to *V. fuscatum,* another wild blueberry species native to the southeastern U.S. Wild tetraploid populations of native *V. corymbosum* occur in abundance on favorable sites as far south as Gainesville in the northern part of the Florida peninsula. Native highbush blueberry plants of similar morphology, ecology, and phenology occur for another 350 km south of where the tetraploid highbush reach their southern limit. Camp [16] called the highbush blueberries from these southern locations *V. fuscatum* but did not have the opportunity to determine whether they were diploid or tetraploid. Vander Kloet [17], who spent several months studying the native blueberries surrounding the Archbold Biological Station in Arcadia, Florida, determined that *V. fuscatum* from south Florida was all diploid. These diploid *V. fuscatum* plants in southwest Florida are evergreen with 1 to 5 major canes, are 2 to 4 m tall, and produce small, black, sweet, juicy berries in abundance [18]. The natural habitats of *V. fuscatum* include sandy flatwoods, bottom lands, and banks along streams or lakes [16]. The morphological characteristics of lanceolate–elliptic leaves, an evergreen growth habit, and small black fruits distinguish *V. fuscatum* from other *Vaccinium* species (Figure 1). Although there is a high level of phenotypic diversity within this species, individual plants from *V. fuscatum* genetically formed a distinct cluster from *V. elliottii* and *V. darrowii* via principal coordinate analysis using expressed sequence tag-simple sequence repeats (EST-SSR) markers [19].

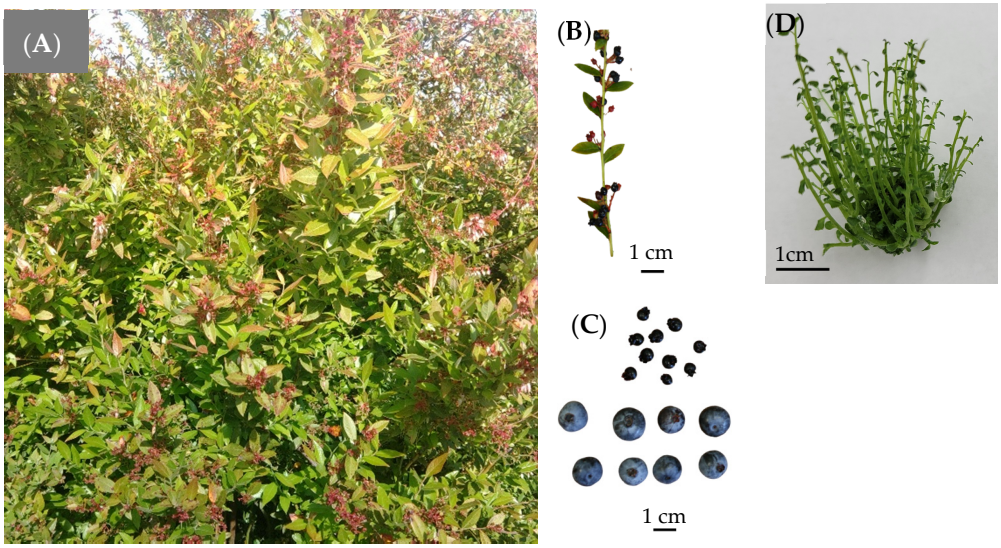


Figure 1. *V. fuscatum* bushes grown at the University of Florida Plant Science Unit in Citra, Florida (**A**); a *V. fuscatum* branch loaded with fruits (**B**); ripe fruits from *V. fuscatum* in the top cluster compared to fruits from SHB at the bottom cluster (**C**); vigorous growth of the *V. fuscatum* clone in tissue culture medium containing 2.3 µM zeatin (**D**).

Since diploid highbush blueberry plants of a similar type extend northward on the coastal plain all the way to the northeastern United States [20], it seems likely that the *V. fuscatum* of south Florida will eventually be found to be conspecific with the diploid highbush blueberry designated as *V. atrococcum* in areas north of Florida. Previously, a diploid *V. fuscatum* clone (reported as *V. atrococcum* Heller) with a high level of field resistance to *Phytophthora cinnamomi* was identified [21]. There is a strong triploid block of producing infertile progenies from crosses between diploid and tetraploid blueberry species [22]. Very few viable hybrids were recovered from *V. fuscatum* and SHB crosses [18]. To circumvent the triploid block, axillary buds from this disease-resistant clone were treated in vivo with colchicine and induced a chimeral plant with some tetraploid branches [23]. Field resistance to phytophthora root rot was retained in the colchicine-induced tetraploid clone [23]. Unfortunately, this valuable *V. fuscatum* clone is not available anymore.

In addition to resistance to *Phytophthora cinnamomi*, *V. fuscatum* is of interest in blueberry breeding because it has the plant architecture of cultivated highbush blueberry, comes from a place that receives essentially no winter chilling, and grows as an evergreen plant. Evergreen production systems for SHB blueberries are established in regions with mild winters such as California, Florida, Hawaii in the U.S., and other countries, including Spain, China, Morocco, Mexico, Argentina, and Australia [24–26]. Low chilling requirements, disease, and pest resistance are required to keep healthy leaves all year round for evergreen production. Blueberry leaf rust (*Thekopsora minna*) is a major fungal pathogen that causes defoliation and yield reduction in evergreen blueberry production in Florida [27] and Australia [28]. In addition to blueberry rust, anthracnose, Septoria, and target spots can also cause premature leaf defoliation and yield reduction in blueberries in Florida. As a Florida native species with an evergreen growth habit, *V. fuscatum* is tolerant of or resistant to multiple blueberry foliar diseases. The utilization of *V. fuscatum* to improve the vigor and resistance of SHB blueberries to foliar diseases is highly desirable. In this study, we identified a robust *V. fuscatum* clone with an evergreen growth habit and good regeneration capability in tissue culture (Figure 1). To overcome the ploidy barrier between this species and SHB, we induced the polyploidy of the *V. fuscatum* clone through in vitro colchicine treatment.

Polyploidy induction through colchicine treatment effectively produced fertile synthetic tetraploids from diploid *V. elliottii* and *V. darrowii*, as was reviewed recently [29]. Several antimitotic agents, including trifluralin, amiprophos methyl, and oryzalin, were used for polyploidy induction [29]. Among these, colchicine was the most widely used for multiple *Vaccinium* species; therefore, it was chosen to induce polyploidy in *V. fuscatum*. Colchicine disrupts spindle formation during mitosis, resulting in cells with an additional set of chromosomes [30]. The optimization of colchicine treatment conditions is necessary to reduce the lethality of treated materials from the cytotoxic effect of colchicine and improve the recovery rate of polyploidy-induced materials. A wide range of colchicine concentrations from 2.5 µM to 12,518 µM was used for polyploid induction of *Vaccinium* species [23,31,32]. The regeneration of polyploidy-induced mutants from colchicine treatment was genotype-specific. For instance, the colchicine concentration at 2.5 µM was found to be optimum for producing synthetic octoploid mutant clones from the NHB cultivar 'Duke' [32], whereas 250 µM colchicine was effective in inducing synthetic tetraploids from *V. elliottii* and *V. darrowii* [33]. Five hundred µM and five thousand µM colchicine concentrations were chosen for this experiment based on previously reported effective colchicine concentrations [31]. Increasing the ploidy level of diploid *V. fuscatum* to tetraploid could potentially improve the efficiency of interspecific hybridization and allele introgression. The objectives of this research are (1) evaluating the efficiency of polyploid induction of *V. fuscatum* in comparison with SHB cultivars, (2) recovering synthetic tetraploid *V. fuscatum* upon colchicine treatment, and (3) characterizing morphological changes in the synthetic tetraploid *V. fuscatum*. The success in inducing polyploids in diploid *V. fuscatum* reported in this study opens an efficient avenue for utilizing this valuable wild blueberry species in blueberry cultivar improvement.

2. Materials and Methods

2.1. Blueberry Plant Materials

In July 2017, softwood cuttings for clonal propagation were taken from 30 diploid highbush blueberry plants growing in southwest Florida. About half of the plants came from a wet forest in southwestern DeSoto County near Ft Myers, Florida. Two plants came from a wetland southeast of Frostproof, Florida, and ten were selected from the wet margins of Lake Istokpoga near Sebring, Florida. The cuttings were rooted and grown in 5-gallon pots of peat. In January 2019, 30 potted plants, one from each genotype, were placed outside with several dozen honeybee hives near the University of Florida entomology department building, Gainesville, FL, where they were isolated from other diploid blueberry plants. One hundred mature berries were harvested from each plant. The seeds were extracted in bulk and germinated in a greenhouse in Gainesville, FL. Approximately fifty of the resulting seedlings were planted at the University of Florida Plant Science Unit in Citra, Florida, in May 2020. In August 2023, soft new tissue was taken from ten plants that had the largest leaves and most upright stature to establish tissue cultures in Tifton, Georgia. Tissue culture was established for six out of the ten plants. *V. fuscatum* clone 'FL 21-1423' was selected for colchicine treatment due to its high vigor in tissue culture (Figure 1D). SHB blueberry cultivars 'Emerald' [34] and 'Rebel' [35] established in tissue culture by Fall Creek Nursery (Lowell, OR, USA) were treated together *V. fuscatum* 'FL 21-1423' to compare the efficiency of polyploidy induction. *V. fuscatum* 'FL 21-1423' was simplified as *V. fuscatum* for the rest of the writings. Due to the presence of a high level of genetic diversity in *V. fuscatum* [19], additional research will be needed to determine the representativeness of this clone for *V. fuscatum*.

2.2. Tissue Culture and Colchicine Treatment

To avoid the high contamination rate from using plant tissue collected directly from the field [36], tissue from new axillary shoots grown in a controlled environment was used to initiate tissue culture. In this process, softwood cuttings from the *V. fuscatum* clone grown in the field were defoliated and washed with mild hand soap by using a paintbrush for 3 min. The cleaned shoots were rinsed with tap water and transferred to a beaker filled with tap water. The shoots were placed on a rack next to the window of the lab with a constant temperature of 24 °C. Once axillary shoots grew to approximately 20 mm long in about a month, they were harvested and defoliated. Harvesting the new shoots growing in the lab minimized the contamination from the field. The shoots were immersed in 50 mL Conical tubes with 30% Clorox and 0.005% Tween-20 and agitated for 20 min. The shoots were transferred to new sterile tubes and rinsed with sterilized water 3 times for 3 min per rinse. The shoots were segmented into single-node segments and placed horizontally in individual 15 mL tubes with 3 mL of woody plant medium (Phytotech, Lenexa, KS, USA) containing 13.7 µM zeatin (Phytotech). The WPM medium was made by adding 3% sucrose (Aldon, Avon, NY, USA) and adjusting the pH to 5.2 with NaOH. 0.8% agar (Sigma Aldrich, St. Louis, MO. USA), which was added before autoclaving at 121 °C for 20 min. Zeatin was supplemented after the medium cooled to 60 °C. To determine the lethality of colchicine treatment, *V. fuscatum* and SHB cultivars 'Rebel' and 'Emerald' were treated with WPM liquid media supplemented with 0 µM, 500 µM, and 5000 µM colchicine (Sigma Aldrich) solution for 48 h. During the treatment, the 50 mL tubes containing the shoots with various colchicine solutions were rocked at 30 rpm on an UltraRock rocking platform (Bio-Rad Laboratories, Hercules, CA, USA). The shoots were washed with sterile deionized water 5 times for 3 min per wash. Single-node stem segments were placed horizontally on the WPM medium with 2.3 µM zeatin for axillary shoot induction. For each genotype/treatment combination, there were three experimental replicates, with each replicate consisting of five single-node segments arranged in one petri dish. The shoot number and shoot length were measured for each regenerated shoot 100 days after the colchicine treatment. To ensure recovery of sufficient polyploidy-induced materials, in a separate experiment, seventy-two nodal segments of 'Rebel' and 'Emerald' were treated with 500 µM colchicine, whereas 200 nodal segments each were treated with 5000 µM colchicine. As for *V. fuscatum*, seventy-nine nodal segments were treated for both colchicine levels. Tissue culture plates were grown in the I-66LLVL biological incubator (Percival Scientific, Perry, IA, USA) at 26 °C with a photon flux density of 40–50 µmoL/m^2/s with 16/8 day and night cycles.

2.3. Rooting the Shoots from Tissue Culture

Shoots of 2 to 3 cm long were separated from the tissue culture clusters and transplanted to the 72-cell propagation tray (https://www.bootstrapfarmer.com, accessed on 8 July 2025) filled with pine bark medium. The propagation tray was enclosed with a clear cover to maintain the high humidity for four weeks in the heated greenhouse set at 18 to 28 °C. Upon root formation, the plantlets were transferred to 1-gallon pots and fertilized as needed with the slow-release 10-10-10 fertilizer (Scotts Miracle-Gro Company, Marysville, OH, USA).

2.4. Flow Cytometry Analysis

Two to three leaves per shoot regenerated in tissue culture were dissected for ploidy level determination. The protocol from CyStain® PI Absolute P staining kit (Sysmex Partec GmbH, Görlitz, Germany) was modified to adapt to the 5–10 mg of tissue harvested from shoot explants. An equal amount of leaf tissue from the target plantlet and the control

was chopped together in 60 µL of extraction buffer for 30 to 60 s in a Petri dish chilled on ice. Five hundred µL of working staining solution (0.5 µL staining buffer, 3 µL propidium iodide (PI) dye, 1.5 µL of RNase, and 495 µL of water) was added to the nuclei extract and incubated for 60 s. The PI-labeled nuclei solution was filtered through a 30 µm CellTrics filter (Sysmex Partec GmbH) and incubated on ice for 10 min before running through the Attune NxT Acoustic Focusing Cytometer (ThermoFisher, Scientific, Walthman, MA, USA). Those with an X-mean confidence interval less than 5% were included in ploidy determination using the following formula. The X-mean value is the mean propidium iodide (PI) fluorescence signal emitted via the PI-labeled nuclei. This value indicates the intensity of the fluorescent signal of the target nuclei. To determine the ploidy level of colchicine-treated *V. fuscatum*, tissue from RE (rabbiteye, $2n = 6x = 72$) was used as a control (Figure 2A,B). To determine the ploidy level of colchicine-treated SHB, diploid *V. fuscatum* was used as the control [37].

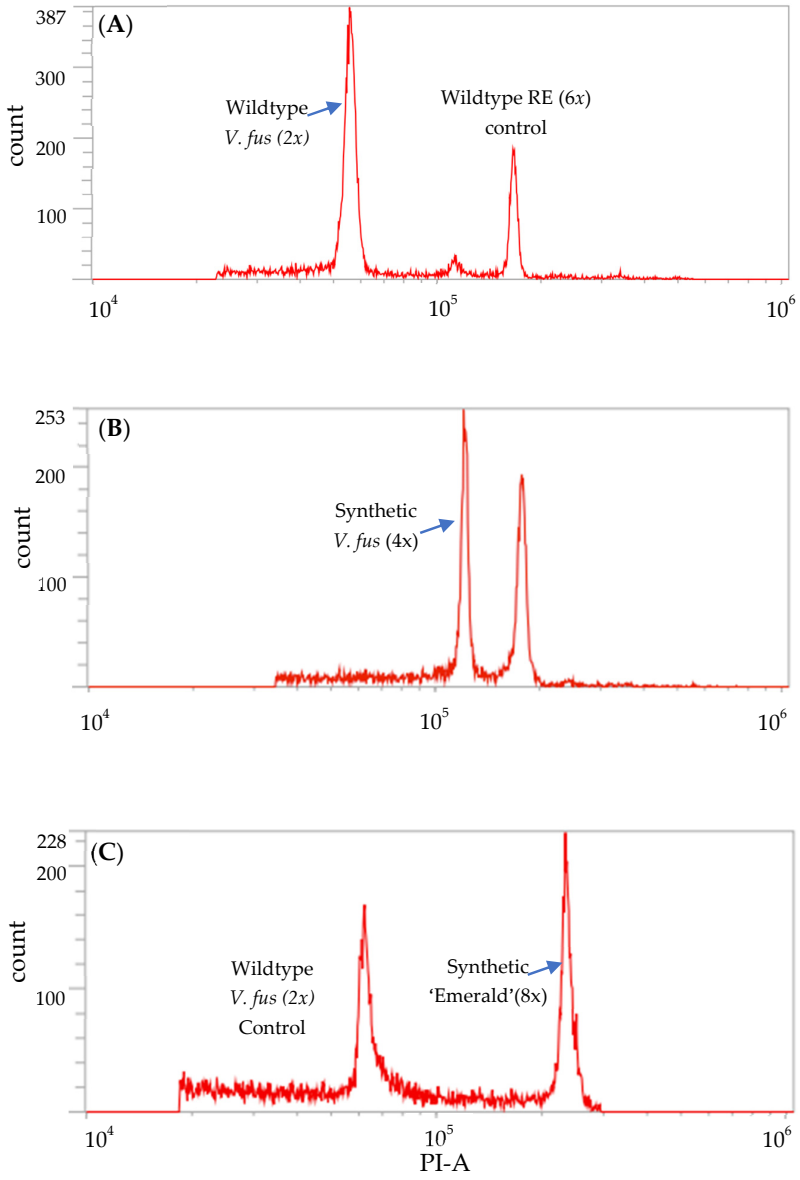


Figure 2. Flow cytometry plots differentiating polyploidy-induced mutant lines from the wildtype controls. (**A**) Nuclei from the wildtype *V. fuscatum* produced a 2*x* peak and RE control produced the 6*x* peak; (**B**) synthetic tetraploid *V. fuscatum* produced a 4*x* peak closely adjacent to the 6*x* peak of the RE control; (**C**) synthetic octoploid 'Emerald' produced an 8*x* peak at the far right of 2*x* peak produced by the wildtype *V. fuscatum* control.

$$\text{Target ploidy level} = \frac{\text{X_mean of target}}{\text{X_mean of control}} * \text{ploidy level of control}$$

The ploidy level of solid synthetic polyploids was confirmed via an additional flow cytometry analysis with the new shoots micro-propagated from the putative shoots.

2.5. Morphological Characterization

Axillary shoots collected from tissue culture were imaged in a sterile laminar flow hood using a digital microscope (Moysuwe, https://www.amazon.com/MOYSUWE-Microscope-Magnifier-Soldering-Compatible/dp/B0CB2J33SB?ref_=ast_sto_dp, accessed on 8 July 2025) before leaf sample collection for flow cytometry analysis. The stem thickness of these shoots was measured via image analysis using APS Assess 2.0 (The American Phytopathological Society, St. Paul, MN, USA). After the tissue-cultured shoots were well established in the greenhouse, leaf samples were collected from eight-month-old plants for stomata, guard cell, and leaf size measurements. For stomata impressions, the third leaf from the top was collected. A thin layer of clear nail polish was applied to the abaxial side of the leaf. Once dried, the stomata impression was lifted from the leaf tissue by applying clear tape. The impression was subsequently loaded onto a microscope slide and viewed under a Nikon Eclipse Si microscope (Nikon Corporation, Tokyo, Japan). Three fields of view were recorded for stomata density from each leaf. Three leaves per genotype were recorded. Each leaf was considered a biological replication. The length and width of the guard cells were recorded from nine stomates with closed pores from each genotype using APS Assess 2.0. For leaf size, images of another twelve mature leaves were imported into the APS Assess 2.0 software to determine leaf width and length. For plant height, the length of the main cane was measured to represent plant height. The number of canes produced by each plant was counted.

2.6. Statistical Analysis

To determine the effect of polyploidy induction on plant morphology, a Student's *t*-test was performed to compare the measurements between the synthetic polyploids and their respective wildtype plants. Statistical significance was determined at $p < 0.05$. Normality tests were performed using the Shapiro–Wilk test, and the datasets were found to be normally distributed.

3. Results and Discussion

For the colchicine lethality experiment, diverse genotypic responses to axillary shoot induction were observed in the WPM medium without colchicine, which was consistent with the previous report on *Vaccinium* species [38]. 'Rebel' produced a callus at the base of the new shoots, whereas callus formation was minimal for 'Emerald' and *V. fuscatum* (Figure 3). Callus formation at the base of the axillary shoot is undesirable in this case since none of the callus tissue further differentiated and produced adventitious shoots [39]. *V. fuscatum* exhibited vigorous growth and produced an average of eight shoots per nodal segment, which was significantly higher than those of 'Rebel' and 'Emerald,' whose average shoots per segment were four (Figure 4A). No significant difference in the axillary shoot length was found among these genotypes without colchicine treatment (Figure 4B). This result confirmed our initial observation of the vigorous shoot growth of this *V. fuscatum* clone while establishing tissue culture from softwood cuttings (Figure 1D). When the nodal segments were treated with 500 µM and 5000 µM of colchicine, the significant suppression of shoot induction and elongation was found in all three genotypes (Figure 3). At a 500 µM colchicine level, *V. fuscatum* produced an average of 4.7 shoots per segment, which was significantly higher than those of 'Rebel' and 'Emerald,' whose average shoot number per

segment was reduced to 0.6 and 0.3, respectively (Figure 4A). In addition, the shoot length of *V. fuscatum* was also significantly longer than 'Rebel' and 'Emerald' (Figure 4B). At the 5000 µM colchicine level, the average number of shoots per nodal segment was reduced to 0.2, 0.3, and 0.9 for *V. fuscatum*, 'Rebel,' and 'Emerald,' which was not significantly different across the genotypes. There was no significant genotypic difference in shoot length at 5000 µM colchicine level, and the average shoot length was reduced to less than 6 mm. In this experiment, although the suppressive effect of colchicine on plant growth was found at both the 500 and 5000 µM levels, *V. fuscatum* survived better than SHB cultivars at the 500 µM colchicine level by producing more vigorous axillary shoots.

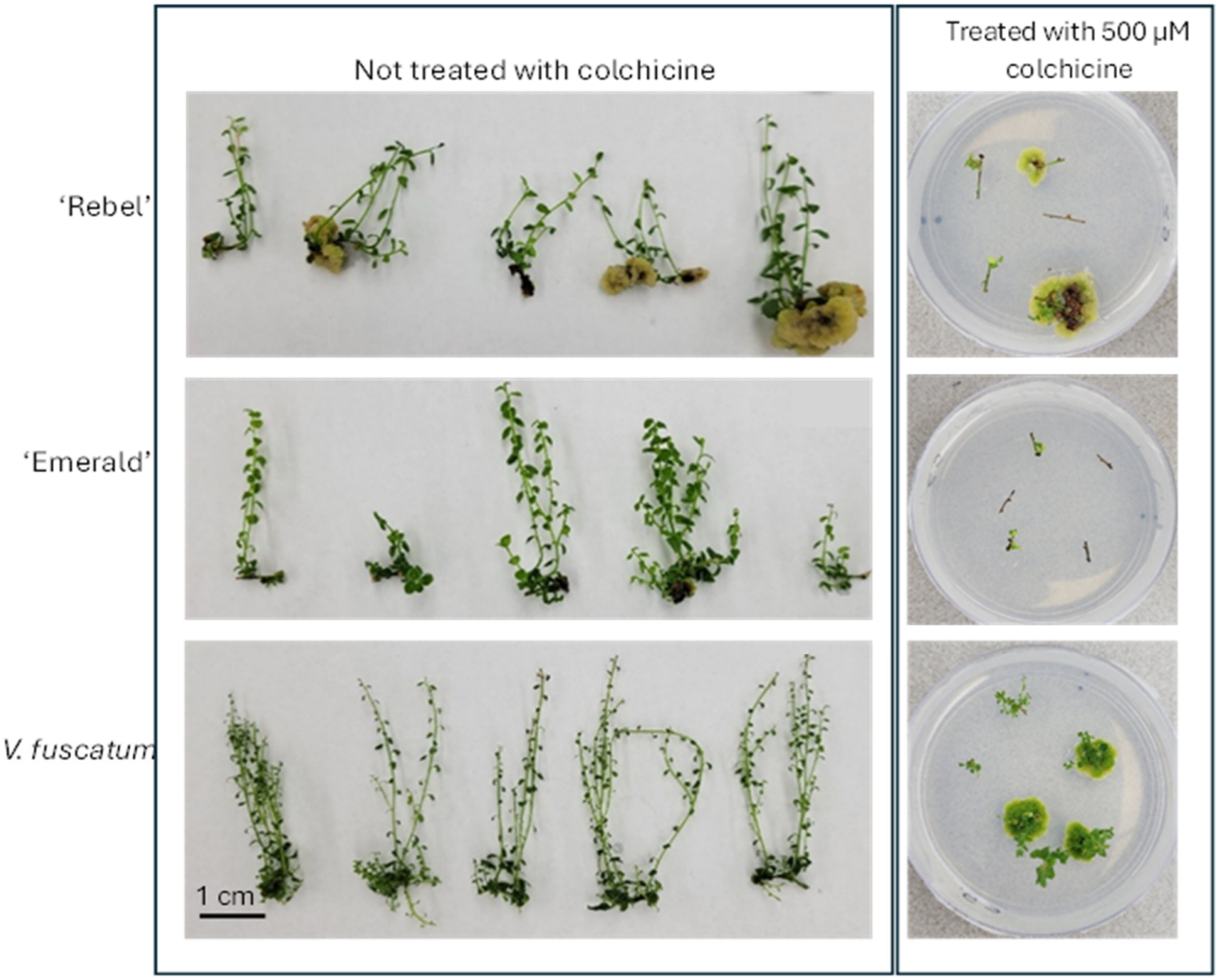


Figure 3. Plantlets from 'Rebel', 'Emerald', and *V. fuscatum* micro-propagated on WPM medium not treated with colchicine (**left** panel) and treated with 500 µM of colchicine (**right** panel). Images of plantlets treated with 5000 µM colchicine were omitted due to the minimum growth of all tested genotypes. Images were taken 100 days after treatment.

Colchicine is a natural tri-cyclic alkaloid extracted from *Gloriosa superba* and *Colchicum autumnale* in the *Liliaceae* family [40]. When actively dividing plant cells are treated with colchicine, cell division is arrested due to the binding of colchicine with tubulins, which interrupts microtubules formation during the metaphase of mitotic cell cycle [41]. The absence of chromosomal pair separation results in the retention of two sets of chromosomes in the mutant cell, i.e., polyploidy induction [42]. The lethality of colchicine depends on the concentration and duration of colchicine treatment [43]. In our experiment, the suppressive effect of colchicine on shoot induction is consistent with the previous findings [44]. The

higher survival rate of *V. fuscatum* suggested that it has a higher tolerance of the mitotic agent compared to the SHB cultivars.

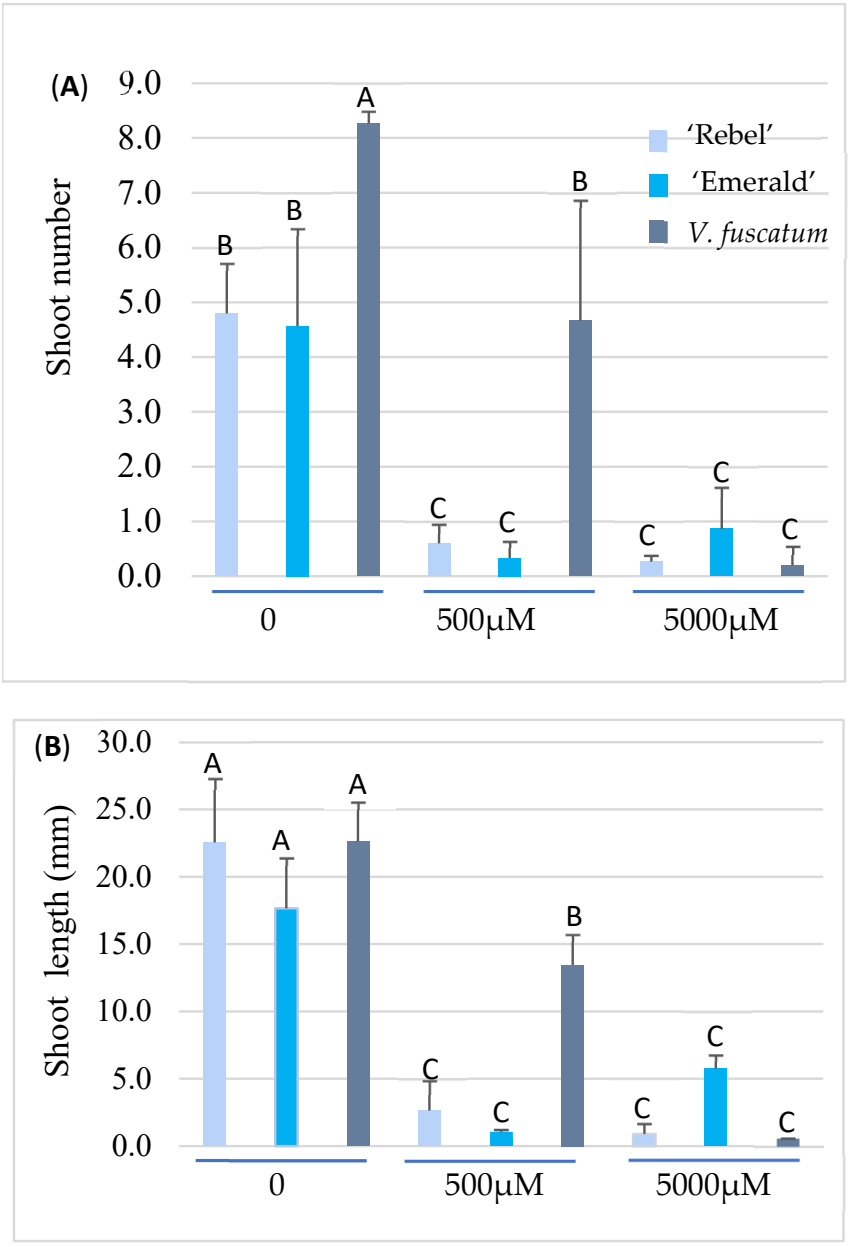


Figure 4. Shoot number per nodal segment (**A**) and length (**B**) measured from axillary shoots induced from colchicine-treated nodal segments. Different letters on top of the bars indicate statistically significant differences at $p < 0.05$.

Due to the low shoot induction rate upon colchicine treatment, the number of nodal segments for colchicine treatment was increased to ensure the recovery of induced polyploids (Table 1).

Table 1. Ploidy levels of shoots regenerated from colchicine-treated nodal segments.

Colchicine Levels	Genotypes	Number of Colchicine-Treated Segments	Number of Regenerated Shoots	Regenerated Shoot per Segment	Number of Shoots Analyzed via Flow Cytometry	Shoot Number at 2*x*	Shoot Number at 4*x*	Shoot Number at 8*x*	Shoot Number of Mixoploidy
500 µM	Rebel	72	30	0.4	30	N/D **	24	3	3
5000 µM	Rebel	200	67	0.3	67	N/D	61	0	6
500 µM	Emerald	72	40	0.6	40	N/D	38	0	2
5000 µM	Emerald	200	76	0.4	76	N/D	73	1	2
500 µM	*V. fuscatum*	79	676	8.6	35 *	22	8	N/D	5
5000 µM	*V. fuscatum*	79	632	8.0	33 *	20	8	N/D	5

* Shoots with an enlarged stem diameter were visually selected for flow cytometry analysis. **: Not detected. These ploidy levels were not detected, and they were not expected from the genetic testing materials.

The average axillary shoots per treated segment ranged from 0.3 to 0.6 for these two cultivars, which was similar to the results of the colchicine lethality experiment (Figure 3). Ploidy levels of all the regenerated shoots from these cultivars were determined via flow cytometry analysis. Three synthetic octoploid 'Rebel' were identified among the thirty regenerated shoots from the 500 µM colchicine treatment. No synthetic octoploid was recovered from the sixty-seven 'Rebel' shoots regenerated from the 5000 µM colchicine treatment. As for 'Emerald,' only one octoploid was identified among the seventy-six regenerated shoots from 5000 µM colchicine treatment, and there was no synthetic octoploid among the forty shoots regenerated from the 500 µM colchicine treatment. The overall success rate of polyploid induction was 3% and 0.9% for 'Rebel' and 'Emerald', respectively. These success rates of polyploid induction were similar to the reported rates of other SHB cultivars, 'Legacy,' 'Duke', and 'Biloxi' [45]. In addition to octoploids, nine and four mixoploids were identified in 'Rebel' and 'Emerald', respectively. Blueberry mixoploids are often discarded due to their genome instability [46]. Since the number of synthetic octoploids was low, we further recovered more synthetic octoploids from these mixoploids through chimera dissociation via leaf organogenesis and axillary shoot induction in a separate study [37].

As for *V. fuscatum*, when the number of treated nodal segments increased to seventy-nine, it exhibited a highly robust growth and completely overcame the suppressive effect from both levels of colchicine treatments. Nine weeks after the colchicine treatment, *V. fuscatum* produced 676 and 632 new shoots at 500 µM and 5000 µM colchicine levels, respectively, which reflected an average of 8.6 and 8 shoots per segment. This level of shoot induction was comparable to the level of shoot induction without colchicine treatment (Figure 3). The exuberant growth of colchicine-treated *V. fuscatum* upon sample size increase indicated that *V. fuscatum* achieves exceptional resilience to detoxify the cytotoxicity of colchicine. Previously, the transcriptome analysis of colchicine-treated plants revealed that, in addition to upregulating genes inhibiting the formation of microtubules, spindles, and chromosomal kinetochores, colchicine also decreased cytokinesis, resulting in reduced cell activity and promoting apoptosis [47]. In addition to applications in plant biology, colchicine has been used to treat inflammatory conditions such as gout disease clinically [48]. Overdose or misuse often resulted in colchicine poisoning among patients [49]. Recently, colchicine cytotoxicity on mammalian cell division and toxin production was reported, and an NEDD8-activating enzyme was shown to mitigate the toxicity effect of colchicine by maintaining cellular integrity [50]. The mechanism of detoxification against colchicine in *V. fuscatum* may involve the differential expression of detoxification enzymes, an altered tubulin binding affinity, and enhanced DNA repair. These potential underlying mechanisms should be further studied since they may provide an alternative therapeutic option for colchicine poisoning.

Regardless, this large amount of shoot induction in *V. fuscatum* made it challenging to screen all of them for flow cytometry analysis. Previously, the enlargement of the stem diameter was reported for polyploid-induced *Vaccinium* species such as *V. elliottii*, *V. darrowii* [33], highbush blueberries, and *V. virgatum* [51]. Consequently, 35 and 33 shoots with thicker stems from 500 µM and 5000 µM colchicine treatments, respectively, were visually selected for flow cytometry analysis (Table 1). A total of 16 synthetic tetraploid shoots were identified, accounting for 24% of the evaluated shoots. Therefore, the preferential selection of *V. fuscatum* shoots with thick stems resulted in a higher recovery rate of synthetic polyploids than the success rates from 'Rebel' and 'Emerald' in our study. This success rate was also higher than the highest success rate of 11% reported in the literature for wild blueberry species *V. myrtillus* [51] and *V. corymbosum* [52]. Therefore, in the case of genotypes highly prolific in shoot induction upon colchicine treatment, it is advisable to speed up the identification of polyploidy-induced mutant lines by visually selecting shoots with thicker stems for flow cytometry analysis.

Producing multiple shoots carrying the same mutation is desirable due to the potential loss of the valuable mutant line to the subsequent micropropagation, rooting, and cultivation processes. These synthetic polyploids are genetically stable and ready for breeding or clonal propagation. However, from the standpoint of creating a diverse genetic resource of *V. fuscatum* at the tetraploid level, it may be more efficient to treat the seed population instead of micro-propagated individual clones and treating them with colchicine. Previously, 0.04% to 5% recovery rates were reported when seeds from *Vaccinium* species were treated with colchicine [44,53–55]. Due to the genetic heterozygosity within *Vaccinium* species [22], the polyploidy-induced mutant lines from individual seeds would carry unique genomic compositions. However, the downside of this alternative method lies in the challenge of differentiating solid polyploidy-induced mutant lines from sectorial, periclinal, and mericlinal chimeras [54].

Polyploidy induction was reported to increase the size and weight of multiple plant organs such as stems, fruits, flowers, and leaves [56]. This type of phenotypic shift has been attributed to the allele dosage effect in the mutant lines [57]. Since only four octoploid shoots were recovered from 'Rebel' and 'Emerald', the stem thickness of these two SHB cultivars was combined to determine the effect of polyploidy. Synthetic octoploid SHB had significantly thicker stems than the wildtype tetraploid SHB (Figure 5A). Similarly, stems of synthetic tetraploid *V. fuscatum* were significantly thicker compared to the wildtype diploid *V. fuscatum* (Figure 5B). These results are consistent with the previous findings of an enlarged stem diameter from colchicine treatment [58–60]. Mixoploids were recovered from both SHB and *V. fuscatum*. The stem thickness of the mixoploids was numerically between the polyploid-induced mutant and wildtype controls for both species (Figure 5). For the SHB samples, the thickness of the mixoploid stems was statistically significantly lower than the synthetic octoploid but similar to the wildtype control. In the case of *V. fuscatum*, the stem thickness of the mixoploid was not statistically different from either the synthetic tetraploid *V. fuscatum* or the wildtype control.

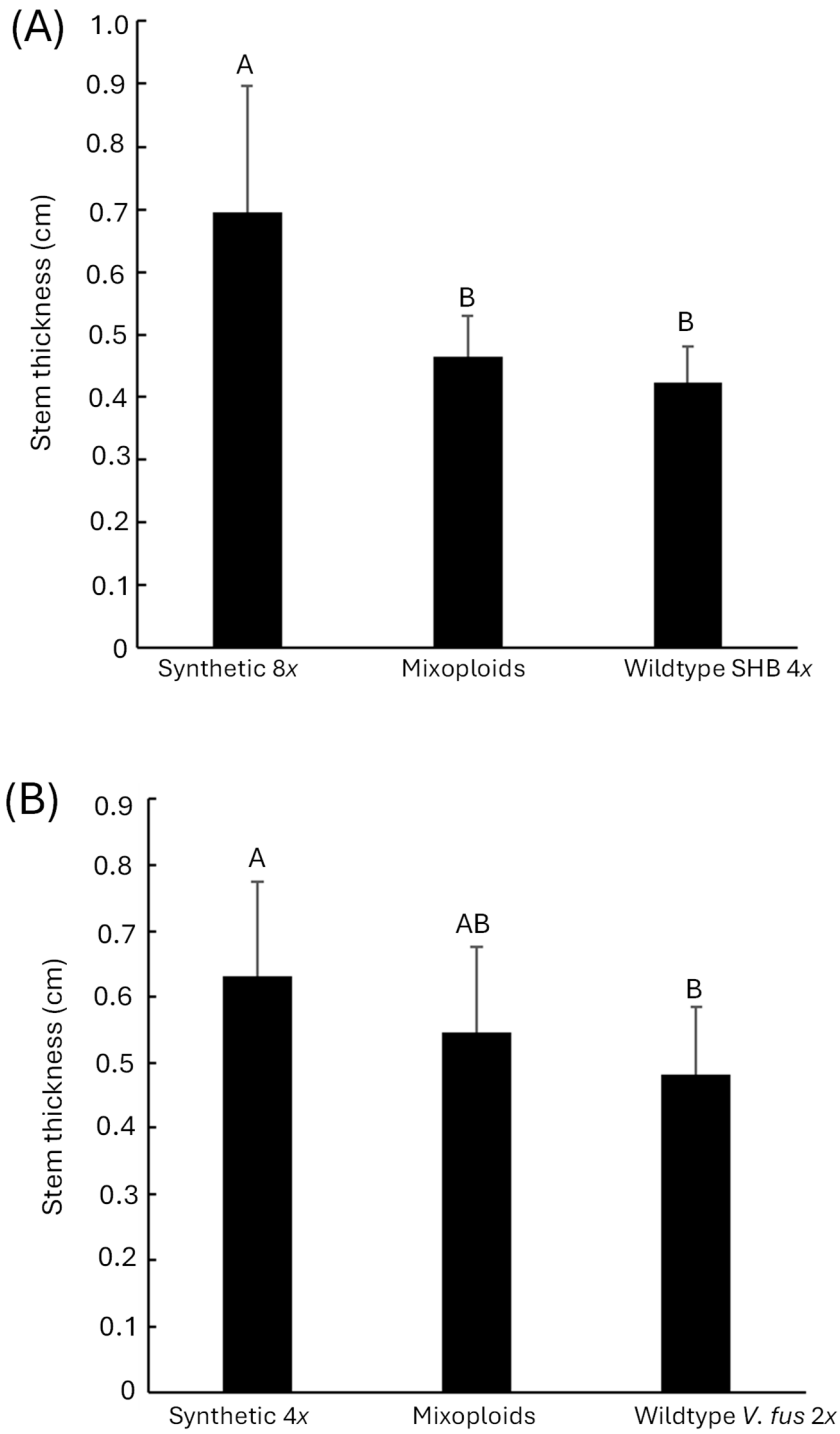


Figure 5. Stem thickness of shoots micro-propagated from polyploidy-induced SHB (**A**) and *V. fuscatum* (**B**) compared to the respective mixoploid and wildtype controls. Measurement was performed after 100 days of transfer. Different letters on the top of the bar indicate statistical difference at $p < 0.05$.

The in vitro micro-propagated shoots were rooted and established in pine bark medium in the greenhouse (Figure 6). Plant morphological characteristics were measured for these plants (Table 2). Upon polyploid induction, both plant height and the number of canes were reduced in all three polyploidy-induced genotypes compared to their respective wildtype controls (Table 2). The differences reached statistical significance except for the number of canes for synthetic tetraploid *V. fuscatum*. These data indicate that the polyploid induction reduced the plant growth of the genotypes included in this

study. Similarly, an increase in the polyploid level was reported to reduce the growth rate of Arabidopsis [61]. As for leaf size, both the synthetic tetraploid *V. fuscatum* and the octoploid 'Emerald' had significantly increased leaf width and length compared to their respective wildtype controls; except that the increment in leaf length for the octoploid 'Emerald' did not reach statistical significance. On the contrary, both the leaf width and length of the synthetic octoploid 'Rebel' were significantly smaller than those of the wildtype control. The increment of leaf length and width in the polyploidy-induced mutants was reported to be associated with an increase in cell size and cell elongation contributed by the 'gigas effect' from genome doubling [62,63]. In our study, the opposite effect of polyploidy induction on the leaf width and length in 'Rebel' indicates a genotype-specific response to polyploidization. This finding conformed with the previous report on perennial giant grasses (*Miscanthus*) in which one out of five polyploidy-induced genotypes had a reduced plant size [64]. This negative response of 'Rebel' could be caused by the retardation of growth development associated with chromosomal doubling [61]. For stomata guard cell size and density, an enlargement of the stomata guard cell size and a reduction in stomata density were observed for both polyploidy-induced SHB and *V. fuscatum* (Table 2, Figure 7). Synthetic octoploid 'Emerald' and 'Rebel' had significantly wider and longer stomata guard cells compared to their respective wildtype controls. Synthetic tetraploid *V. fuscatum* had significantly longer stomata guard cells than the diploid wildtype, whereas the numerical increment in guard cell width in the synthetic tetraploids did not reach statistical significance. Polyploidy-induced *V. darrowii* and *V. elliottii* also reported to have longer guard cells [33,54]. Statistically significant reduced stomata density was found in polyploidy-induced lines of both *V. fuscatum* and 'Emerald' (Table 2 and Figure 7), whereas the reduction in stomata density in 'Rebel' did not reach statistical significance. Previously, a larger guard cell size was found to moderately increase water use efficiency and drought tolerance in *Z. Mays* [65]. A reduction in stomata density was reported in other polyploidy-induced highbush blueberries [52]. Stomata are small pores bound by a pair of guard cells on the leaf surface. They regulate water and CO_2 exchange in response to the environmental cues [66]. A reduction in stomata density was found to be associated with increased drought tolerance in wheat [67,68] and photosynthesis in rice [69]. As the frequency and severity of drought events are intensified due to global climate change [70], testing the drought tolerance and photosynthetic efficiency of the synthetic polyploids in the future will inform the utility of these synthetic mutants in improving drought tolerance in blueberries.

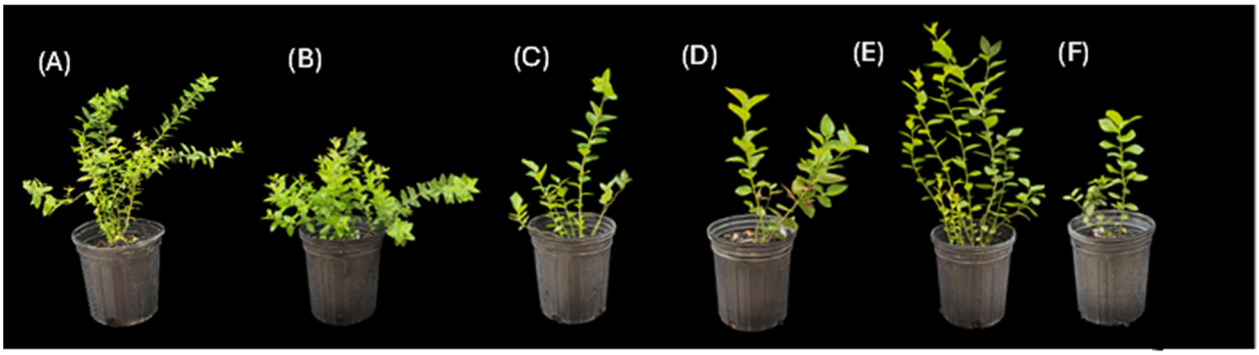


Figure 6. Images of a diploid *V. fuscatum* wildtype plant (**A**), a synthetic tetraploid *V. fuscatum* plant (**B**), a tetraploid wildtype 'Emerald' plant (**C**), a synthetic octoploid 'Emerald' plant (**D**), a tetraploid wildtype 'Rebel' plant (**E**), and a synthetic octoploid 'Rebel' plant (**F**) rooted from tissue-cultured explants and grown in 1-gallon pots for eight months.

Table 2. Plant morphological characteristics measured for wildtype and polyploidy-induced plants grown in 1-gallon pots. Different letters beside the numbers indicate statistical difference at $p < 0.05$.

Genotype	Ploidy Level	Plant Height (cm)	Number of Canes	Leaf Width (cm)	Leaf Length (cm)	Guard Cell Width (µm)	Guard Cell Length (µm)	Stomata Density (no./mm^2)
V. fuscatum control	2*x*	36.5 ± 2.4 ^{a}	3.8 ± 2.2 ^{a}	1.5 ± 0.3 ^{a}	3.3 ± 0.5 ^{a}	11 ± 1.3 ^{a}	20 ± 0.7 ^{a}	367 ± 162 ^{a}
Synthetic *V. fuscatum*	4*x*	29.1 ± 3.6 ^{b}	2.6 ± 1.1 ^{a}	1.9 ± 0.2 ^{b}	3.7 ± 0.4 ^{b}	13 ± 0.7 ^{a}	24 ± 0.6 ^{b}	204 ± 63 ^{b}
Emerald control	4*x*	44.2 ± 12.4 ^{a}	4.3 ± 1.0 ^{a}	3.1 ± 0.3 ^{a}	4.5 ± 0.4 ^{a}	11 ± 0.4 ^{a}	19 ± 0.7 ^{a}	351 ± 199 ^{a}
Synthetic Emerald	8*x*	25.5 ± 5.1 ^{b}	1.8 ± 0.5 ^{b}	3.6 ± 0.5 ^{b}	4.9 ± 0.6 ^{a}	15 ± 0.4 ^{b}	28 ± 0.9 ^{b}	180 ± 87 ^{b}
Rebel control	4*x*	38.3 ± 6.1 ^{a}	4.0 ± 1.4 ^{a}	2.9 ± 0.4 ^{a}	5.0 ± 0.6 ^{a}	10 ± 0.4 ^{a}	17 ± 0.7 ^{a}	193 ± 50 ^{a}
Synthetic Rebel	8*x*	17.8 ± 5.6 ^{b}	2.0 ± 0.8 ^{b}	2.0 ± 0.2 ^{b}	3.1 ± 0.2 ^{b}	12 ± 0.5 ^{b}	20 ± 0.6 ^{b}	113 ± 18 ^{a}

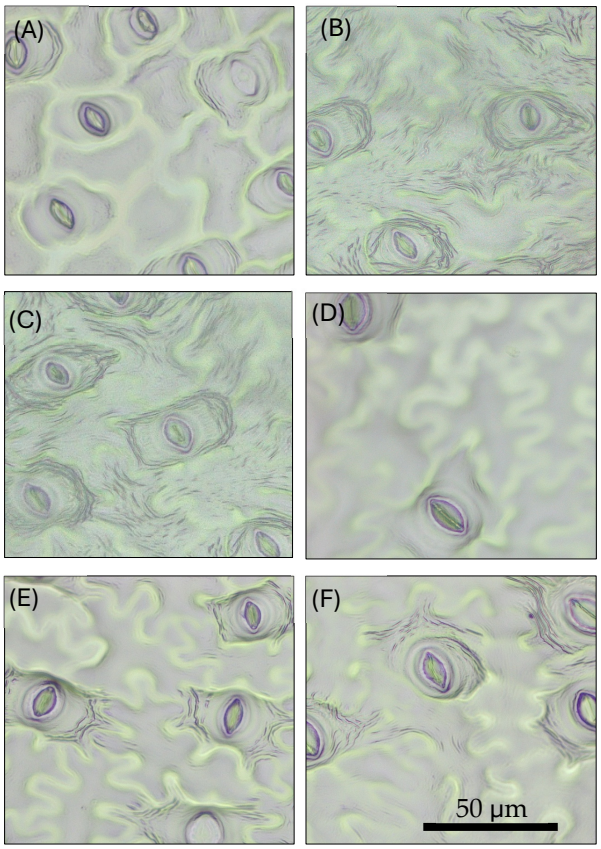


Figure 7. Stomata impressions under microscope. (**A**) diploid *V. fuscatum* wildtype control; (**B**) synthetic tetraploid *V. fuscatum*; (**C**) tetraploid 'Emerald' wildtype control; (**D**) synthetic octoploid 'Emerald'; (**E**) tetraploid 'Rebel' wildtype control; (**F**) synthetic octoploid 'Rebel'.

4. Conclusions

Polyploidy induction through colchicine treatment successfully-induced tetraploid *V. fuscatum* from a wild diploid clone. The high vigor of shoot induction of *V. fuscatum* upon colchicine treatment not only enabled the highly efficient screening of polyploidy-induced mutant lines but could also be useful for studying the mechanism of detoxifying the cytotoxicity of colchicine. The synthetic tetraploid *V. fuscatum* reflected morphological changes, including an increased stem thickness, an increased stomata guard cell size, and a reduced stomata density. These characteristics may increase drought tolerance and photosynthetic efficiency in the synthetic tetraploid *V. fuscatum*. From the perspective of breeding, it is expected that the synthetic tetraploid *V. fuscatum* will produce viable pollen and egg cells that can be used for interspecific hybridization with highbush blueberries. As a perennial bush, blueberries experience one to two years of a juvenile stage before flowering. We will continue to study the synthetic tetraploid *V. fuscatum* concerning its flowering,

fruiting, seed setting, and crossing efficiency with highbush blueberries. In addition, there is a potential fruit size increase in the synthetic *V. fuscatum* from chromosomal doubling. Evaluating the fruit size, yield, and quality will further inform the breeding value of the synthetic *V. fuscatum*. These efforts will contribute to the expansion of the genetic diversity of cultivated blueberries to meet the challenges of blueberry production.

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