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SHORT COMMUNICATIONS

Protoplast isolation systems in blueberry (*Vaccinium corymbosum*) and mortiño (*Vaccinium floribundum*): a novel and efficient approach

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Abstract
The *Ericales* encompass a diverse group of plants, including commercially significant species such as persimmon, blueberries, kiwifruits, Brazil nuts, argan, and azalea. However, to date, no research has been conducted on the isolation of protoplasts in blueberry species such as *Vaccinium corymbosum* L. and *Vaccinium floribundum* Kunth (mortiño). The use of protoplasts for in vitro propagation has become a powerful tool to overcome sexual incompatibility barriers between plant species or genera and to transfer genes for resistance to diseases, pests, herbicides, and other stress factors, thereby enabling the production of a large number of high-quality hybrid plants. Consequently, the isolation of a substantial quantity of protoplasts and the establishment of an efficient regeneration protocols are essential prerequisites for the successful advancement of modern botany. Nonetheless, the establishment of efficient protoplast-based systems remain a challenge for numerous crop plants. The present work outlines the state-of-the-art of protoplast isolation systems within the *Ericales* order. Furthermore, we have successfully established the first and highly efficient system for the isolation of blueberry protoplasts from leaves employing an enzymatic solution comprising 1% cellulase, 1.5% macerozyme, and 0.3% pectinase at incubation for 28 h resulting in a yield of 5.95×10^4 protoplasts (FW). Additionally, callus induction was achieved in mortiño leaf explants by using semi-solid Woody Plant Medium supplemented with 2.5 mg/L of 2,4-Dichlorophenoxyacetic acid. Subsequently, we developed a highly efficient system for the isolation of mortiño protoplast, employing an enzymatic solution containing 2.5% cellulase, 3% macerozyme, and 0.3% pectinase atincubation for 5 hours resulting in a yield of 1.05×10^5 protoplasts (FW). Here, we have reported for the first time a highly efficient system for protoplast isolation in blueberries, making a significant milestone in the advancement of new plant breeding technologies for these species and other related crops.

Keywords Protoplasts isolation systems · Blueberry (*Vaccinium corymbosum* L) · Mortiño (*Vaccinium floribundum* Kunth) · *Ericales* order

Introduction

The order *Ericales* comprises 346 genera and approximately 12,000 species distributed across 21 families. This order includes woody trees and shrubs that often thrive in nutrient-poor or acidic soils, with flowers typically exhibiting symmetrical corollas with loosely fused petals (Rose et al. 2018). Among the economically significant members of this order is the genus *Vaccinium*, which includes species such as *Vaccinium corymbosum* L. (blueberry) and *Vaccinium floribundum* Kunth (mortiño). The genus *Vaccinium*, belonging to the Ericaceae family, encompasses approximately 450 species primarily distributed across the Northern Hemisphere, the Andes Mountains, and South Africa (Wrońska et al., 2018). These species are typically found at elevations ranging from 1,500 to 4,700 m above sea level, where temperatures range between 4 and 17 °C (Meléndez & Flor, 2021). Blueberries are the most widely cultivated species in this genus due to their frost resistance and high

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commercial demand, largely driven by their classification as a superfood (Yavorska, 2016). They are recognized for their high content of polyphenols, flavonoids, phenylpropanoids, sugars, lipids, vitamins, and minerals, making them highly valuable for human nutrition (Meléndez et al. 2021). Similarly, mortiño has gained scientific and commercial interest due to its antioxidant and anti-inflammatory potential. However, its adaptation to controlled cultivation, particularly in greenhouse environments, remains challenging (Cobo et al. 2016; Llivisaca-Contreras et al. 2022). Despite the growing demand for these species, in vitro propagation and regeneration studies on mortiño remain scarce (Rache & Pacheco, 2010). In contrast, blueberries have demonstrated successful commercial production via in vitro culture techniques (Debnath 2007; Castro 2016; Georgieva and Kondakova 2021), yet research on protoplast isolation and regeneration in this species remains largely unexplored. Both blueberries and mortiño face significant challenges in traditional vegetative propagation, with pests and diseases posing risks to plant development, fruit production cycles, and overall yield quality (Becerra et al. 2017). Given these limitations, micropropagation and protoplast isolation techniques present promising alternatives to facilitate large-scale disease-free plant production and enhance fruit quality.

Protoplasts, which are plant cells with enzymatically removed cell walls, are extensively used in tissue culture-based breeding strategies due to their capacity to regenerate into whole plants. They are totipotent, highly sensitive, and versatile, making them valuable for diverse applications in plant biotechnology (Jadán & Dorca, 2019). Several factors critically influence successful protoplast isolation, including enzyme type, enzyme concentration, explant type, enzymolysis duration, osmotic pressure, and purification method (Chen et al. 2023). Optimizing these parameters is essential to achieving efficient cell wall digestion and protoplast viability. The isolation, culture, and fusion of protoplasts is a pivotal area in plant research, offering applications such as single-cell cloning, somatic hybridization via protoplast fusion, genetic transformation through DNA uptake, and cellular studies of membranes and organelles (Veilleux et al. 2004). Given these advantages, protoplast-based methods have emerged as powerful tools in plant breeding. Unlike conventional breeding and transgenic CRISPR/Cas9 approaches, gene editing via transient transformation and protoplast regeneration allows precise genome modifications within a single clonal generation while avoiding the integration of foreign DNA into the host genome (Reed and Bargmann 2021). This technique enables greater control over donor DNA delivery, resulting in enhanced precision and efficiency. While protoplast-based systems have been successfully implemented for plant regeneration in related *Vaccinium* species, such as Rhododendron (Tu et al. 2009), Mountain Laurel (Hasegawa et al. 2005), Tea Plant (Xu et al. 2021), and Strawberry Tree (Choury et al. 2018), their application in blueberries remains insufficiently explored.

The present study establishes a highly efficient protoplast isolation system from in vitro cultures of blueberry and mortiño. To achieve this, a comprehensive literature review on protoplast isolation in *Ericales* species was conducted, enabling the selection of optimal source materials and enzymatic digestion conditions. We successfully developed a high-efficiency protoplast isolation system for blueberry leaves resulted in a protoplast yield of 5.95×10^4 protoplasts/g fresh weight (FW). Additionally, we established a highly efficient protoplast isolation system for mortiño calli yielded 1.05×10^5 protoplasts/g FW demonstrating its effectiveness for protoplast extraction in this species. To the best of our knowledge, this study presents the first detailed and efficient protoplast isolation method for blueberry and mortiño plants.

Materials and methods

In vitro establishment and multiplication of blueberry shoots

Blueberry plants (*Vaccinium corymbosum* L.), Biloxi variety, cultivated under greenhouse conditions in Bolívar, Carchi, Ecuador, were collected for the study. The plants were subjected to a phytosanitary control protocol twice per week using Copper sulfate pentahydrate (SKUL-27®), a systemic fungicide, at a concentration of 2 mL/L. Young, slightly lignified stems approximately 5 cm in length, each containing two axillary buds, were selected. All leaves were removed prior to surface disinfection, which was performed as follows: 20 min under running water, 15 min in a 2% soap solution, 1 min in 70% ethanol, 5 min in 3% sodium hypochlorite, and 5 min in 0.4% (270 g/L) SKUL-27®. Between each step, explants were rinsed with sterile distilled water to eliminate residual disinfectants. Following disinfection, the explants were inoculated onto Woody Plant Medium (WPM) (Lloyd and McCown 1981) supplemented with 3 mg/L 6-benzylaminopurine (6-BAP), 30 g/L sucrose, and 7 g/L agar, following the methodology described by Castro et al., (2016) (Fig. 1a).

Protoplasts isolation from in vitro blueberries leaves

One-month-old in vitro blueberry leaves were weighed (1 g) and finely chopped to increase the contact surface area with the enzymatic solution. The fragmented leaves were suspended in 5 mL of BH3 medium containing 0.7 M mannitol,

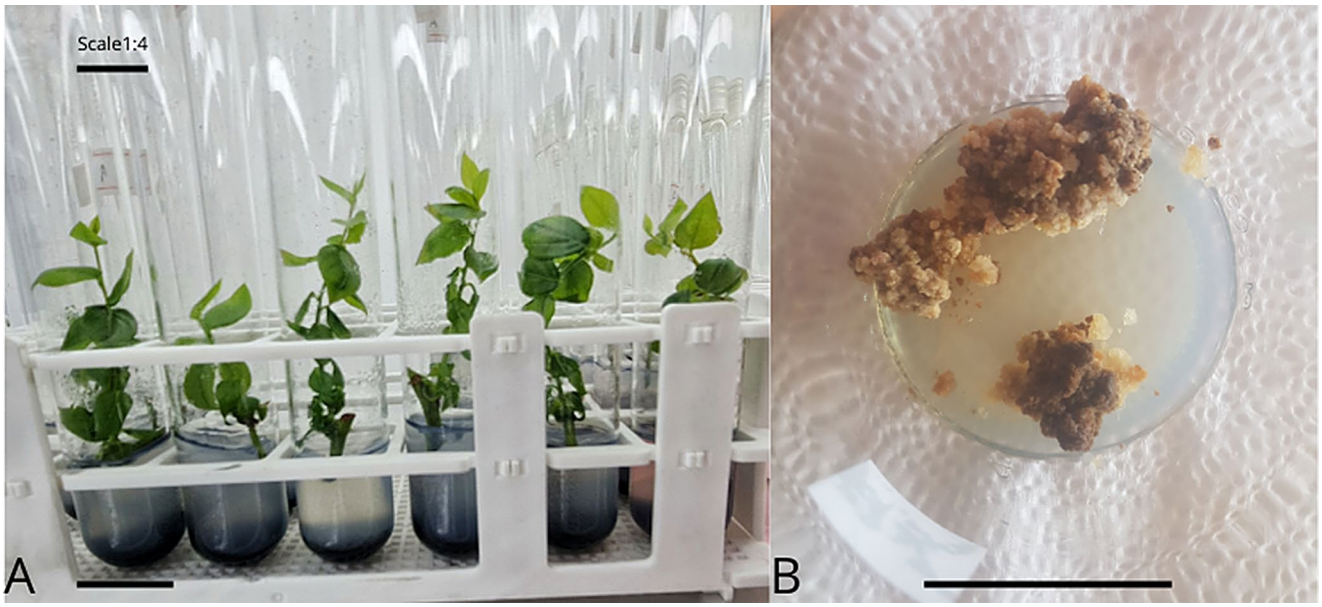

Fig. 1 **(A)** In vitro blueberry leaves **(B)***Mortiño* calli at 21 days after inoculation (dai). Scale bar = 1:4

adjusted to pH 5.7. Subsequently, 5 mL of enzymatic solution was added, comprising 0.7 M mannitol, 12 mM calcium chloride, 6 mM MES, and 13 mM monobasic sodium phosphate, along with specific enzymes according to the treatment conditions outlined in Table 2. No sterile water washing was performed before the enzymatic procedure. To enhance the extraction of the middle lamella, vacuum pressure was applied to disrupt surface tension and ensure complete submersion of the plant material. The samples were subsequently incubated in darkness at 27 °C with continuous agitation at 90 rpm. Protoplast yield was quantified every two hours.

Mortiño callus induction and multiplication

Leaves from wild *mortiño* (*Vaccinium floribundum* Kunth) plants were collected from Pasochoa, Pichincha, Ecuador. The youngest leaves were selected, and their edges were trimmed to obtain fragments of approximately 1 cm². The plant material was then subjected to a surface disinfection procedure as follows: 20 min under running water, 10 min in a 2% soap solution, 3 min in 70% ethanol, 5 min in 3% sodium hypochlorite, and 5 min in 0.4% (270 g/L) SKUL-27®. Between each step, explants were rinsed with sterile distilled water to remove residual disinfectants. Following disinfection, the explants were inoculated onto Woody Plant Medium (WPM) (Lloyd & McCown 1981) supplemented with 2.5 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D). This culture medium was used for both callus induction and multiplication stages. Callus tissue was collected 21 days after inoculation, as by this time, it had exhibited sufficient growth to provide the quantity required for protoplast isolation (Fig. 1b). For each isolation assay, 0.5 g of callus tissue was weighed.

Protoplasts isolation from mortiño calli

For the isolation of protoplasts from *mortiño* callus, 3 mL of 0.7 M BH3 culture medium and 3 mL of enzymatic solution were used. No sterile water washing was performed before the enzymatic procedure. The enzymatic solution consisted of 0.7 M mannitol, 12 mM calcium chloride, 6 mM MES, and 13 mM monobasic sodium phosphate, with specific enzymes added according to the treatment conditions described in Table 3. A total of 0.5 g of in vitro callus tissue was treated with the enzymatic solution. The samples were incubated in darkness at 27 °C with continuous agitation at 95 rpm for 10 h. The concentration of isolated protoplasts was quantified every hour using a hemocytometer.

Statistical analysis

A randomized experimental design was used for the study. Statistical analysis was performed using one-way ANOVA, and mean comparisons were performed using Tukey's test ($P \leq 0.05$). Data analysis was carried out using InfoStat 2017 software for Windows.

Results and discussion

In recent years, species within the order *Ericales*, particularly those of the *Vaccinium* genus, have gained substantial agro-commercial interest. However, their propagation

remains challenging both in field and greenhouse conditions, emphasizing the need for efficient and cost-effective cultivation systems to enhance yield. Protoplast culture represents a promising alternative for advancing studies in plant physiology and genetic improvement (Reed and Bargmann 2021). Successful protoplast isolation requires the enzymatic digestion of the plant cell wall, a process highly dependent on the optimization of enzyme composition and digestion parameters to maximize protoplast yield and viability. In addition, the selection of plant material is a critical factor in protoplast isolation. Leaf mesophyll and cell suspensions are commonly used explants, with juvenile tissues being particularly responsive due to their enhanced mitotic activity (Roca and Mroginski 1991). Additionally, meristematic aggregates have been reported to yield higher numbers of protoplasts due to their active cell division (Jiang et al. 2013). The efficiency of enzymatic digestion is influenced by species-specific variations in cell wall composition, particularly in lignin, cellulose, and pectin content, necessitating tailored enzyme concentrations and digestion durations (Suárez, 2020). Although higher enzyme concentrations may improve protoplast yield, they can also compromise cell viability, requiring a fine balance between enzyme concentration and digestion time (Zhi-Xin 2011). Therefore, establishing an optimized protoplast isolation system for each genotype is essential (Castro, 2017).

Analyzing species related to *Vaccinium corymbosum* (blueberry) and *Vaccinium floribundum* (mortiño) within the *Ericales* order, we identified that *Arbutus unedo* exhibited an efficient protoplast isolation system using a combination of Cellulase R-10 (2%) and Macerozyme (1%). This protocol was optimized with prolonged incubation periods and increased concentrations of Cellulase RS and mannitol when older leaves were utilized as explants (Choury et al. 2018). Similarly, studies on *Rhododendron* spp. demonstrated high protoplast yields with an enzymatic cocktail containing 2% cellulase, in combination with pectinase at concentrations of 0.5%, 1%, and 2% (Tu et al. 2009). Building upon this knowledge, we conducted a comprehensive review of protoplast isolation systems for species within the order *Ericales* to establish an optimized system for *Vaccinium corymbosum* and *Vaccinium floribundum* (Table 1). Our findings corroborate that both enzyme concentration and digestion time are pivotal factors influencing protoplast yield and viability. Most effective systems typically employed a 2% cellulase cocktail, often combined with pectinase (0.5–2%) or macerozyme (0.6–1%). Notably, cellulase concentrations exceeding 3% consistently resulted in decreased yield, which we attribute to the toxic effects of the enzyme (Tu et al. 2009). Our study evaluated four enzymatic treatments for in vitro blueberry leaves, consisting of different concentrations of cellulase, macerozyme, and pectinase (Table 2). No significant protoplast release was observed before 20 h of digestion (Fig. 2a). Protoplast concentration was quantified every two hours from 20 to 34 h, revealing that the highest yield (5.95×10^4 protoplasts/g fresh weight) was achieved using treatment Ta2 (1% cellulase, 1.5% macerozyme, 0.3% pectinase) after 28 h of digestion. Statistical analysis confirmed significant differences among treatments (Fig. 2b). For *mortiño*, protoplast isolation was conducted from callus tissue induced from leaf explants cultured on semisolid Woody Plant Medium (WPM) supplemented with 2.5 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D). Three enzyme combinations with varying concentrations and incubation times were tested (Table 3). Protoplasts were quantified hourly for 10 h (Fig. 2c), with significant differences observed between treatments. The highest yield (1.05×10^5 protoplasts/g fresh weight) was obtained with treatment Ta3 (2.5% cellulase, 3% macerozyme, 0.3% pectinase) after 5 h of digestion (Fig. 2d).

The monitoring frequency of protoplast yield differed between *mortiño* and blueberry due to the structural

Table 1 Summary of the protoplast isolation systems in the order *Ericales*

Protoplast isolation									Protoplast purification	
Reference	Species	Type of explant	Enzyme combination		Osmotic regulator	pH	Yield ($10^6 n$/g FW)	digestion time	Spin speed	Spin time
(Tu et al. 2009)	Rhododendron (*Rhododendron* spp)	In vitro leaf	Cellulase R-10 Pectinase Y-23	1%, 2% and 3% 0.5%, 1% and 2%	0.9 M Mannitol	5.0 5.5 6.0	1.47	14 h	1000 rpm	1000 rpm
(Hasegawa et al. 2005)	Mountain laurel (*Kalmia latifolia*)	In vitro leaf	Cellulase R-10 Macerozyme	2% 1%	1 M Mannitol	5 a 6		15 h	210 x *g*	210 x *g*
(Xu et al. 2021)	Tea plant (*Camellia sinensis*)	In vitro leaf	Cellulase Macerozyme	1.5% 0.6%	0.4 M Mannitol	5.7		10 h	200 x *g*	200 x *g*
(Choury et al. 2018)	Strawberry tree (*Arbutus unedo*)	*Ex vitro* leaf	Cellulase R-10 Macerozyme	2% and 4% 1%	0.8 M Mannitol	5.0	61.3	16 h		

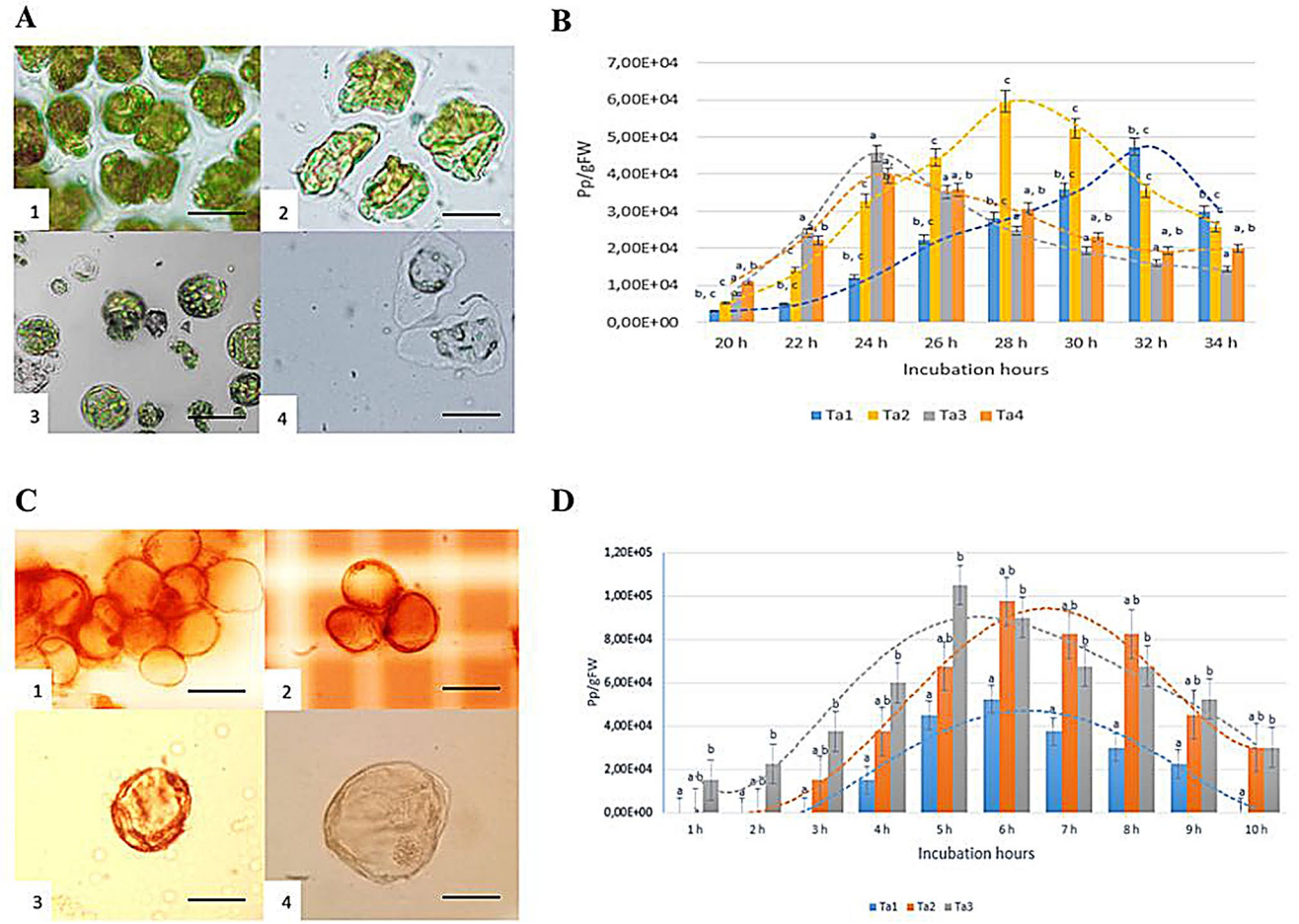

Fig. 2 **(A)** In vitro blueberry leaf protoplasts at 100× magnification. Images were taken at different incubation times: (1) 14 h, (2) 18 h, (3) 28 h, and (4) 34 h. Scale bar = 50 µm. **(B)** Mean concentration curves ($n = 3$) of isolated blueberry protoplasts treated for 34 h. Data are presented as the mean of three biological replicates, with error bars representing standard deviations (SD). Different letters (a–d) indicate statistically significant differences between treatments at $p < 0.05$, based on Tukey's test. Pp/g FW = protoplasts per gram of fresh weight. **(C)** *In vitro mortiño* callus protoplasts. Images were taken at different incubation times: (1) 3 h, (2) 4 h, (3) 5 h, and (4) 6 h. Scale bar = 50 µm. **(D)** Optimal treatment conditions were determined for *mortiño* callus. Data are presented as the mean of three biological replicates, with error bars indicating standard deviations (SD). Different letters (a–b) indicate statistically significant differences between treatments at $p < 0.05$, based on Tukey's test

properties of their tissues. The friable nature of *mortiño* callus necessitated shorter enzymatic digestion times, justifying hourly monitoring, whereas blueberry leaf tissues exhibited greater resistance to enzymatic degradation, requiring quantification at two-hour intervals. These findings emphasize the importance of tailored enzymatic digestion parameters for each species. Furthermore, *V. corymbosum* protoplasts were derived from in vitro leaves, whereas *V. floribundum* protoplasts originated from in vitro callus tissue. This methodological distinction holds significant implications for future studies involving protoplast fusion between these species, as their protoplasts exhibit distinct visual characteristics: blueberry protoplasts appear green, while *mortiño* callus protoplasts are translucent yellow. Beyond optimizing protoplast yield, osmotic regulation was a key consideration in our study. The inherent fragility of protoplasts necessitates precise control of osmotic regulators to maintain isotonic conditions, preventing both rupture due to hypotonic stress and plasmolysis under hypertonic conditions (Bargmann, 2021). Our system employed Cell Protoplast Wash (CPW) solution containing 13% mannitol, which has been demonstrated to enhance osmotic stability in plants. Mannitol serves multiple roles, including membrane hydration, free radical scavenging, and structural stabilization (Patel and Williamson 2016).

Overall, our findings establish a valuable framework for future research on protoplast fusion and genetic improvement of *Vaccinium* species. Further investigations will focus on optimizing culture conditions to enhance protoplast viability and regeneration, as well as exploring somatic hybridization approaches to improve the agronomic traits of these economically significant crops.

Conclusions

This study represents a significant advancement in protoplast isolation protocols in the order *Ericales*. We successfully established, for the first time, an optimized protoplast isolation method for blueberry and mortiño, providing a foundation for protoplast-based research in these species. Our findings highlight the most efficient treatments: blueberry leaf-derived protoplasts achieved the highest yield using 1% cellulase, 1.5% macerozyme, and 0.3% pectinase with a 28-hour digestion, whereas mortiño callus-derived protoplasts reached maximum yield using 2.5% cellulase, 3% macerozyme, and 0.3% pectinase after 5 h. The establishment of these protocols opens new avenues for genetic improvement and somatic hybridization in *Vaccinium* species. Protoplast fusion between blueberry and mortiño presents an exciting opportunity to enhance traits such as disease resistance, stress tolerance, and fruit quality. The distinct coloration of protoplasts from each species offers a practical advantage for monitoring fusion events, facilitating further studies in hybrid development. Additionally, mortiño, a species native to the high Andes, holds ecological importance and faces conservation challenges (Cobo et al. 2016). Our findings contribute to the broader effort of integrating biotechnology into sustainable agriculture and conservation programs. Future research should explore the applications of protoplast technology in *Ericales* crop improvement, including genetic transformation, cytoplasmic hybridization, and synthetic polyploidization. These approaches will be instrumental in developing resilient cultivars capable of withstanding climate change while maintaining high nutritional and agronomic value.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s42535-025-01201-5.

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Data availability The authors confirm that the data supporting the findings of this study are available within the article.

Declarations

Conflict of interest The authors declare no conflict of interest.

References

Becerra C, Defilippi B, France A, González A, Hirzel J, Morales CG, Pedreros A, Riquelme J, Robledo P, Uribe H (2017) Manual de manejo agronómico del arándano. Boletín INIA, 98

Castro A (2016) Mejora de la propagación *in vitro* de *Vaccinium corymbosum* y evaluación de la actividad antioxidante en arándanos comerciales. *Máster En Biología Molecular, Celular y Genética.*, 40

Castro Hinojosa CW (2017) *Obtención de protoplastos del mesófilo de hojas de tomate (Solanumlycopersicum Mill.) mediante digestión enzimática*.http://repositorio.espe.edu.ec/jspui/handle/21000/13773

Chen K, Chen J, Pi X, Huang LJ, Li N (2023) Isolation, purification, and application of protoplasts and transient expression systems in plants. Int J Mol Sci 24(23):16892. https://doi.org/10.3390/ijms242316892PMID: 38069215; PMCID: PMC10706244

Choury Z, Meschini R, Dell'Orso A, Fardusi MJ, Mugnozza GS, Kuzminsky E (2018) Optimized conditions for the isolation of mesophyll protoplasts along the growing season from *Arbutus unedo* and their use in single cell gel electrophoresis. Plant Cell Tissue Organ Cult (PCTOC) 132(3):535–543. https://doi.org/10.1007/s11240-017-1349-6

Cobo MM, Gutiérrez B, Torres AF, de Torres M L (2016) Preliminary analysis of the genetic diversity and population structure of mortiño (*Vaccinium floribundum*Kunth). Biochem Syst Ecol 64:14–21. https://doi.org/10.1016/j.bse.2015.11.008

Debnath SC (2007) Propagation of *Vaccinium in Vitro*: a review. Int J Fruit Sci 6(2):47–71. https://doi.org/10.1300/J492v06n02_04

Georgieva M, Kondakova V (2021) *In vitro* propagation of *Vaccinium corymbosum L*. Bulg. J Agric Sci 27(2):323–327

Hasegawa S, Meguro A, Toyoda K, Nishimura T, Kunoh H (2005) Drought Tolerance of tissue-cultured seedlings of Mountain Laurel (Kalmia latifolia L.) Induced by an endophytic actinomycete II. Acceleration of Callose Accumulation and Lignification. Actinomycetologica 19(1):13–17. https://doi.org/10.3209/saj.19.13

Jadan M, Dorca-Fornell C (2019) Propagation methods in Babaco plants (*Vasconcella x Helbornii*). Trop Plant Res 6(1):37–45. https://doi.org/10.22271/tpr.2019.v6.i1.007

Jiang F, Zhu J, Liu H (2013) Protoplasts: a useful research system for plant cell biology, especially dedifferentiation. Protoplasma. https://doi.org/10.1007/s00709-013-0513-z

Llivisaca-Contreras SA, León-Tamariz F, Manzano-Santana P, Ruales J, Naranjo-Morán J, Serrano-Mena L, Chica-Martínez E, Cevallos-Cevallos JM (2022) Mortiño (*Vaccinium floribundum*Kunth): an underutilized superplant from the Andes. Horticulturae 8(5):358. https://doi.org/10.3390/horticulturae8050358

McCown BH, Lloyd G (1981) Woody Plant Medium (WPM)—A Mineral nutrient formulation for microculture of Woody Plant species. HortScience 16:453–453

Meléndez M, Estefanía FR, Esther SP, Vásquez W, Racines-Oliva M (2021) *Vaccinium* spp.: Karyotypic and phylogenetic characteristics, nutritional composition, edaphoclimatic conditions, biotic factors and beneficial microorganisms in the rhizosphere. Scientia Agropecuaria 12:109–120. https://doi.org/10.17268/sci.agropecu.2021.013

Patel TK, Williamson JD (2016) Mannitol in plants, Fungi, and plant-fungal interactions. Trends Plant Sci 21(6):486–497. https://doi.org/10.1016/j.tplants.2016.01.006

RacheL, Pacheco J (2010) *In vitro* propagation of mature plants of *Vaccinium meridionale (Ericaceae)*. Acta Bot Brasilica 24:1086–1095. https://doi.org/10.1590/S0102-33062010000400024

Reed KM, Bargmann BOR (2021) Protoplast Regeneration and Its Use in New Plant Breeding Technologies. *Frontiers in Genome*

Editing, *3*.https://www.frontiersin.org/article/https://doi.org/10.3389/fgeed.2021.734951

Roca W, Mroginski LA (1991) *Cultivo de tejidos en la agricultura: Fundamentos y aplicaciones*.https://cgspace.cgiar.org/handle/10568/53954

Rose JP, Kleist TJ, Löfstrand SD, Drew BT, SchönenbergerJ, Sytsma KJ (2018) Phylogeny, historical biogeography, and diversification of angiosperm order *Ericales* suggest ancient neotropical and east Asian connections. Mol Phylogenet Evol 122:59–79. https://doi.org/10.1016/j.ympev.2018.01.014

Suárez Padrón IE (2020) *Cultivo de Tejidos Vegetales*. Ed. Fondo Editorial Universidad de Córdoba.https://repositorio.unicordoba.edu.co/handle/ucordoba/2553

Tu Y, Xiong L, Leng B (2009) The Studies on Technique Conditions of Protoplast Isolation and Purification of Three Species of Rhododendron. *Journal of Jiangxi Normal University (Natural Sciences Edition)*, *2*

Veilleux RE, Compton ME, Saunders JA (2004) Use of protoplasts for plant improvement. In *Plant Development and Biotechnology* (pp. 213–224)

Wrońska-Pilarek D, Szkudlarz P, Bocianowski J (2018) Systematic importance of morphological features of pollen grains of species from Erica (Ericaceae) genus. PLoS ONE 13:e0204557. https://doi.org/10.1371/journal.pone.0204557

Xu X, Zhu H, Ren Y, Feng C, Ye Z, Cai H, Wan X, Peng C (2021) Efficient isolation and purification of tissue-specific protoplasts from tea plants (*Camellia sinensis* (L.) O. Kuntze). Plant Methods 17(1):84. https://doi.org/10.1186/s13007-021-00783-w

Yavorska NY et al (2016) Microclonal propagation of the varieties of highbush blueberry *Vaccinium corymbosum* l. Biotechnologia Acta 9:30–37. https://doi.org/10.15407/biotech9.05.030

Zhi-xin L (2011) Preparation of Protoplast from leaves of Northland Blueberry. J East Liaoning Univ