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In Vitro Propagation of *Peumus boldus* Mol, a Woody Medicinal Plant Endemic to the Sclerophyllous Forest of Central Chile

Francesca Guerra [1], Loreto Badilla [2], Ricardo Cautín [3] and Mónica Castro [1,*]

[1] Laboratorio de Propagación, Escuela de Agronomía, Facultad de Ciencias Agronómicas y de los Alimentos, Pontificia Universidad Católica de Valparaíso, La Palma S/N, Quillota 2260000, Chile; francesca.guerra@pucv.cl
[2] Altoverde Paisajismo, Quillota 2260000, Chile; altoverde@123.cl
[3] Laboratorio de Especies Leñosas, Escuela de Agronomía, Facultad de Ciencias Agronómicas y de los Alimentos, Pontificia Universidad Católica de Valparaíso, La Palma S/N, Quillota 2260000, Chile; ricardo.cautin@pucv.cl
* Correspondence: monica.castro@pucv.cl

Abstract: *Peumus boldus* Mol (*P. boldus*), a Chilean endemic tree species occurring in sclerophyllous or evergreen forests, has historically played a significant role in indigenous ancestral medicine. Recently, *P. boldus* has garnered renewed attention, spurred by the growing interest in its leaves and bark. These parts contain a wealth of bioactive agents, including alkaloids, flavonoids, essential oils, and potent antioxidant properties attributed to their high phenolic compound content. However, the species' regeneration within its native habitat has been hindered by a confluence of factors such as climate change, agricultural activities, and shifts in land use. Coupled with the seeds' low germination rate and protracted emergence period, the necessity to develop large-scale propagation methods to bolster *P. boldus* population numbers has become increasingly evident. Furthermore, the widespread use of *P. boldus* for medicinal purposes renders it vulnerable to overexploitation, thereby underscoring the need for a comprehensive mass propagation protocol to support conservation efforts. Thus, the main objective of this study was to formulate an in vitro protocol for mass regeneration of *P. boldus*. The explants excised from nodal sections demonstrated an average survival rate of 74%, while the application of 6-benzylaminopurine (4.44 µM) yielded an average shoot length of 5.9 cm. In vitro shoot rooting achieved a success rate of 80% using perlite supplemented with indole-3-butyric acid (9.84 µM). During the acclimation phase, in vitro rooted plants displayed a remarkable 100% survival rate at the 30-day mark after being transplanted into a substrate consisting of perlite and peat in a (1:1 *v*/*v*) ratio.

Keywords: in vitro cultivation; ecological restoration; boldo; woody plant; native flora; micropropagation; medicinal plant

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1. Introduction

Peumus boldus Mol (*P. boldus*), an endemic tree species native to Chile, belongs to the *Monimiaceae* family and is the sole representative of the *Peumus* genus. Geographically, its distribution extends from the northern end of Tongoy Bay (30°20′ South latitude) in the Coquimbo region to the southern banks of the Damas River in Osorno (41°20′ South latitude) [1–3]. It thrives primarily within the sclerophyllous or evergreen forest ecosystem, reaching heights of up to 20 m. It is characterized by a slow growth rate and a spherical crown adorned with coriaceous, dark green leaves. The leaves possess a rough texture on the upper surface and a soft, light yellow-green hue on the underside. They emit an aromatic fragrance, are notably thick, and feature short petioles [3–5].

This species has a long history of use in indigenous ancestral medicine [6]. In recent years, it has garnered increasing interest due to the leaves' capacity to promote digestion, act as a diuretic, and aid in addressing liver and gallbladder disorders. In addition, the

bark of this species contains bioactive compounds such as alkaloids, flavonoids, essential oils, and potent antioxidants, all attributed to its remarkable concentration of phenolic compounds [7–12].

The ecological status of the species' native habitat has been affected by climate change, agricultural activities, and shifts in land use, resulting in a reduced regenerative capacity of the *P. boldus* population. In addition, the species' seeds exhibit a low germination rate, while its seedlings are characterized by a prolonged emergence period [13–15]. Over the years, this species has been propagated by cuttings and seeds with success rates of 16% and 44%, respectively [13,16,17]. There are few antecedents for the micropropagation of this species. For the multiplication stage, between 2.46 and 4.92 µM of BAP (6-benzylaminopurine) has been used, reaching four shoots per nodal section [18]; on the contrary, when epicormic shoots have been used as explants, the culture medium must be supplemented with 1.11–3.33 µM of BAP. In addition, for the rooting stage, it has been indicated that rhizogenesis occurred slowly after 30 days [19]. Considering this background, for the micropropagation of this species, factors such as the type and concentration of growth regulators in the base culture medium must be taken into account; they manage to promote organogenesis in micropropagation [20]. This is why the use of auxins and cytokinins play an important role, since auxins promote cell growth and rhizogenesis, while cytokinins promote cell division as an important process for the generation of shoots and in the massive multiplication of a species [20–22].

In this context, the need to establish comprehensive large-scale propagation methods has become crucial to increasing the population size and contributing to the conservation of this medicinal plant. The use of in vitro mass propagation for habitat restoration has emerged as a viable strategy, given that conventional methods involving cuttings or seeds fall short in guaranteeing substantial production and the creation of pathogen-free germplasm [23–25]. Therefore, the aim of this study was to develop and implement a protocol for mass in vitro regeneration of *P. boldus*.

For this study, four *Peumus boldus* mother plants propagated by seeds were used in order to maintain genetic variability and thus obtain viable populations for their conservation. For this, explants of nodal sections were collected to establish an in vitro propagation protocol, where the effect of three culture media was evaluated in the in vitro establishment stage. For the in vitro multiplication stage, different concentrations of BAP were used to determine the multiplication rate of the species. On the other hand, IBA (indole-3-butyric acid) was used in different concentrations to determine the effect on rooting. In addition, rooted vitroplants were used for their acclimatization to determine survival at this stage. This work is expected to contribute to knowledge of the massive multiplication of this endemic medicinal species of Chile.

2. Materials and Methods

2.1. Plant Material under Study

For this study, seeds of *P. boldus* were collected in 2018 from the Melón mountain, located in the northwest of the Valparaíso region (latitude 32°30′ and 32°46′ South and 71° and 71°13′ West longitude). The climate in this area is characterized as warm temperate and supra-temperate, with a semi-arid moisture regime [26]. Seeds were sown on a propagation bench, and the resulting seedlings were transplanted into 10 L containers filled with peat and coconut fiber substrate (1:1 v/v). These initial plants were used to establish the mother stock of the species, currently maintained in a cold greenhouse. To mitigate potential genotype-related effects on the results, four plants were randomly selected and designated as Bol1, Bol2, Bol3, and Bol4. These designated plant specimens were used for the subsequent in vitro assays detailed below.

2.2. Disinfection of Plant Material

Six young shoots of *P. boldus* were randomly harvested from each of the four designated plant specimens (Bol1, Bol2, Bol3, and Bol4). Disinfection procedures were applied

individually to each shoot, treating them separately. After removing the leaves, nodal explants measuring 1.5 cm in length, each containing two axillary buds, were carefully excised. The explants were washed under running water for 5 min, then thoroughly rinsed with a sponge and 1 mL of Tween 20. The explants were then placed in an Erlenmeyer flask and washed under a continuous stream of running water for 15 min. Subsequently, the nodal explants were disinfected in a solution of 1% sodium hypochlorite, which was supplemented with the antioxidant's ascorbic acid (2838.97 µM) and citric acid (2602.49 µM), along with 1 mL of Tween 20. The mixture was agitated continuously for 10 min. This was followed by a series of five rinses with sterile distilled water under a laminar flow hood.

2.3. In Vitro Establishment

In this assay, the effect of three different culture media (M1, M2, and M3) on the survival rate (%) of four designated *P. boldus* specimens (Bol1; Bol2; Bol3; Bol4) was evaluated using nodal section explants. The following media were used: M1—WPM (Lloyd and McCown, 1980) [27], M2—MS (Murashige and Skoog, 1962) [28], and M3—DKW (Driver and Kuniyuki, 1984) [29]. Each medium was supplemented with 1 g L^{-1} polyvinylpyrrolidone, 0.75 µM thiamine, and 30 g L^{-1} sucrose; the pH was adjusted to 5.8 ± 0.1, followed by the addition of 6.5 g L^{-1} agar. The culture media were autoclaved (121 °C for 15 min). The flasks containing the nodal sections were transferred to the growth chamber at a temperature of 25 ± 1 °C, with a photoperiod of 16:8 h (light/dark cycle) under fluorescent lamps Philips TL-D 36W/54 brand (cold white light 400–700 nm with a PPFD of 9.96 µmol m^{-2} s^{-1}). Evaluation was performed at 15 days, factoring in the ratio of viable explants to the total number of explants used per treatment. The evaluation included explant contamination and oxidation rates (marked by a dark brown discoloration that progresses to black, culminating in the death of the plant). This assay was designed using a completely randomized 4 × 3 factorial arrangement. Each treatment used 10 explants with three replicates for a total of 360 samples. Percentages were natural logarithm-transformed. A two-way analysis of variance (ANOVA) and a component analysis were performed for the data. The means were compared with a Tukey test ($p \leq 0.05$) using the Minitab statistical software (Minitab Inc., State College, PA, USA).

2.4. In Vitro Shoot Multiplication

In this proliferation assay, the influence of different concentrations of 6-benzylaminopurine (BAP) incorporated into the basal MS culture medium (Murashige and Skoog, 1962) was evaluated. This basal medium showed the most favorable results during the establishment phase. Four boldo plants (Bol1; Bol2; Bol3; Bol4) were evaluated along with three concentrations of BAP (BAP1: 0 [control], BAP2: 2.22 µM; BAP3: 4.44 µM). Two-centimeter shoots from the establishment phase were used for the experiment (Figure 1). These shoots were grown in the MS basal medium, containing its standard salts (macro- and microelements) and vitamins, and supplemented with 30 g L^{-1} sucrose. The shoots were placed in a growth chamber for 45 days at a controlled temperature of 25 ± 1 °C, with a photoperiod of 16:8 h (light/dark cycle) under fluorescent lamps Philips TL-D 36W/54 brand (Cold white light 400–700 nm with a PPFD of 9.96 µmol m^{-2} s^{-1}). The experiment included the evaluation of the number of shoots, shoot height, and proliferation rate (number of shoots/initial shoot). The experiment was designed in a completely randomized fashion with a 4 × 3 factorial arrangement. Eight shoots per treatment were used in three replicates, resulting in a total of 288 samples. A two-way analysis of variance (ANOVA) and a component analysis were performed for the data. The means were compared with a Tukey test ($p \leq 0.05$) using the Minitab statistical software.

2.5. In Vitro Rooting and Acclimation

In this assay, the effect of three concentrations of indole-3-butyric acid (IBA) (IBA1: 0 (control), IBA2: 4.92 µM, and IBA3: 9.84 µM) on the rooting (%) of *Peumus boldus* shoots (Bol1; Bol2; Bol3; Bol4) obtained at the multiplication stage was evaluated. The culture was carried out in 300 mL vessels containing 50 mL of perlite. The perlite was moistened

with 30 mL of MS basal growth medium, which contained its standard salts and vitamins, along with the corresponding treatments. The culture vessels were autoclaved at 121 °C for 15 min. The rooting percentage (number of shoots with roots of 0.5 cm length/total number of shoots) was evaluated. The experimental design followed a completely randomized scheme using a 4 × 3 factorial arrangement. Six shoots were used per treatment with three replicates each, for a total of 216 samples. A two-way analysis of variance (ANOVA) and a component analysis were performed for the data. The means were compared with a Tukey test ($p \leq 0.05$) using the Minitab statistical software. Percentages were natural logarithm-transformed.

Figure 1. Two-centimeter boldo shoots used during the in vitro proliferation stage. Scale bar 1 cm.

During the acclimation phase, the rooted shoots were moved to a cold greenhouse to undergo a process of hardening and adaptation. In preparation for this transition, the relative humidity was gradually decreased over 15 days. The survival rate (%) of plants during the acclimation process was evaluated at 30 days of growth (number of living plants/total number of plants subjected to acclimation).

3. Results

3.1. In Vitro Establishment

Figure 2a shows the percentage of survival of explants of *P. boldus*. The growth medium variable did exhibit an effect ($p \leq 0.05$) on the treatments: *P. boldus* explants' survival reached 74% ($p \leq 0.05$) in the MS growth medium (M2), while it was 55% in the M1 medium and 56% in the M3 medium (Figure 2a). In contrast, the correlation between the designated plant variable (Bol1, Bol2, Bol3, Bol4) and the growth medium variable (M1, M2, M3) was not significant. Moreover, there was no correlation ($p > 0.05$) between explant survival (%) and the designated plant variable.

Furthermore, variable oxidation rate (marked by a dark brown discoloration that progresses to black, culminating in the death of the plant) stood at 16% in the basal MS growth medium (M2), which was notably lower than the rates of 27% and 24% recorded in treatments M1 (WPM) and M3 (DKW), respectively ($p \geq 0.05$) (Figure 2b). Fungal contamination ranged from 5% to 12% (Figure 2c) ($p \leq 0.05$), and bacterial contamination ranged from 4% to 9% (Figure 2d), with MS medium (M2) once again showing the lowest percentage. In contrast, there was no statistically significant correlation between the designated plant variable (Bol1, Bol2, Bol3, Bol4) and the growth medium variable (M1, M2, M3) for oxidation, fungal contamination, and bacterial contamination ($p \geq 0.05$).

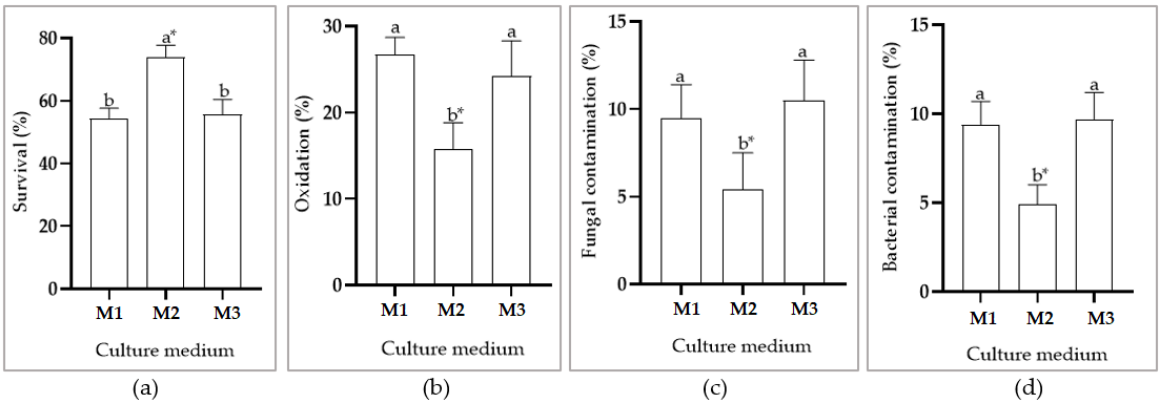

Figure 2. In vitro establishment of *P. boldus* explants. (**a**) Explant survival (%); (**b**) oxidation (%); (**c**) fungal contamination (%); (**d**) bacterial contamination (%). M1: WPM basal medium; M2: MS basal medium; M3: DKW basal medium. * The data in the bar (mean ± standard deviation) with different letters, are significantly different. Tukey test ($p \leq 0.05$).

3.2. In Vitro Shoot Multiplication

Figure 3a shows *P. boldus* shoots cultured for 45 days in the MS growth medium demonstrated increased longitudinal growth (height) in all treatments conducted (Figure 3a). Notably, supplementing the growth medium with 4.44 µM BAP resulted in an average shoot length of 5.9 cm ($p \leq 0.05$) (Figure 3b). In contrast, at concentrations of 0 and 2.22 µM BAP, the average shoot lengths were lower, measuring at 4.1 cm and 4.9 cm, respectively (Figure 3b). In contrast, there was no significant correlation between the designated plant variable (Bol1, Bol2, Bol3, Bol4) and the BAP concentration variable (BAP1, BAP2, BAP3) regarding the average height of boldo shoots. Furthermore, the designated plant variable exhibited no correlation with shoot height ($p > 0.05$).

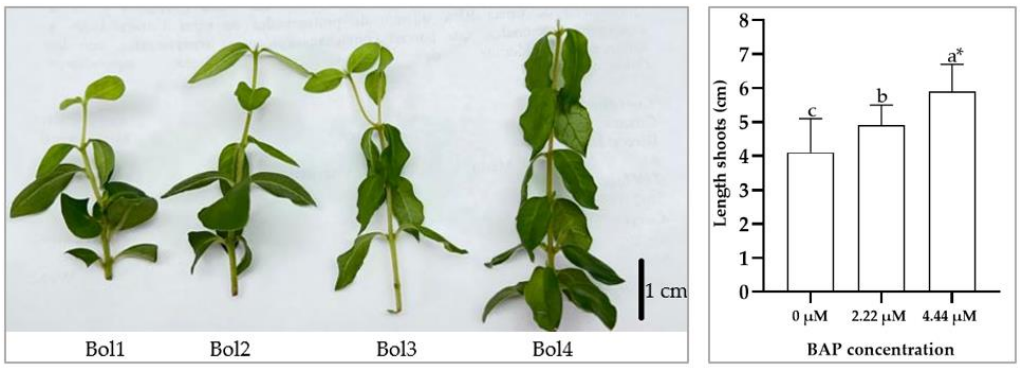

Figure 3. Effect of different BAP concentrations. (**a**) Explant length per treatment; (**b**) *P. boldus* shoots in in vitro proliferation. T1: 0 (control); T2: 2.22 µM BAP; and T3: 4.44 µM BAP. * The data in the bar (mean ± standard deviation) with different letters are significantly different. Tukey test ($p \leq 0.05$). Scale bar: 1 cm.

Furthermore, for the variable number of shoots, different BAP concentrations did exert a substantial effect ($p \leq 0.05$), resulting in an average shoot count of 3.2 upon supplementing the MS growth medium with 4.44 µM BAP (Figure 4a). In contrast, no discernible correlation was observed between the designated plant variable and the BAP concentration variable concerning shoot count. Similarly, the designated plant variable exhibited no significant effect on shoot count ($p > 0.05$).

Regarding the proliferation rate, different BAP concentrations did manifest noteworthy effects ($p \leq 0.05$), with the growth medium supplemented with 4.44 µM BAP yielding a proliferation rate of 4.5 shoots (Figure 4b). In contrast, no apparent correlation emerged

between the designated plant variable and the BAP concentration variable (Bap1, Bap2, Bap3), in relation to the proliferation rate, and no evident correlation was identified between the proliferation rate and the designated plant variable (Bol1, Bol2, Bol3, Bol4).

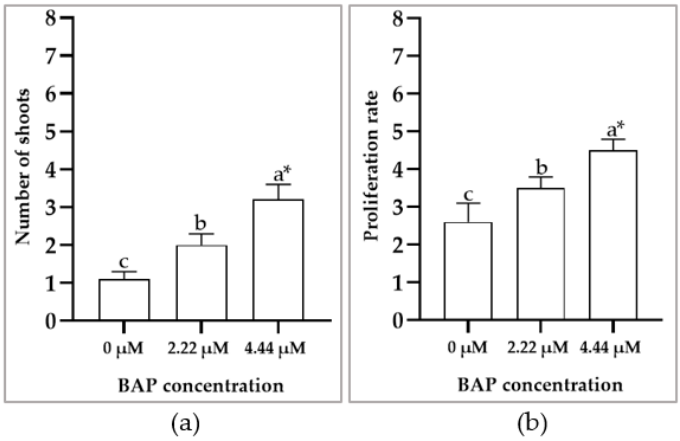

Figure 4. Response of boldo shoots with different concentrations of BAP in the proliferation stage. (**a**) Number of *P. boldus* shoots in in vitro proliferation. (**b**) Proliferation rate of *P. boldus* shoots. T1: 0 (control); T2: 2.22 µM BAP; and T3: 4.44 µM BAP. * The data in the bar (mean ± standard deviation) with different letters are significantly different. Tukey test ($p \leq 0.05$).

3.3. In Vitro Rooting and Acclimation

The analysis of variance indicated no correlation between the designated plant variable and the IBA concentration variable ($p = 0.71$) concerning rooting. Furthermore, the designated plant variable exerted no significant effect ($p = 0.18$) on the rooting (%) of *Peumus boldus* shoots. Nevertheless, the different IBA concentrations did exert an impact ($p \leq 0.05$) on the rooting (%) of *P. boldus* shoots.

Figure 5 illustrates the results obtained by using different IBA concentrations to influence the rooting rate in *P. boldus* vitroplants. The most pronounced effect on rooting was observed in treatment T3 (Figure 5a), wherein the MS growth medium was supplemented with 9.84 µM IBA ($p \leq 0.05$), resulting in an attainment of 85% rooting. On the other hand, when the basal medium was supplemented with 4.92 µM IBA (T2), a rooting rate of 53% was achieved ($p \leq 0.05$). In contrast, with no IBA supplementation in the basal medium (T1), the rooting rate was a modest 17% ($p \leq 0.05$).

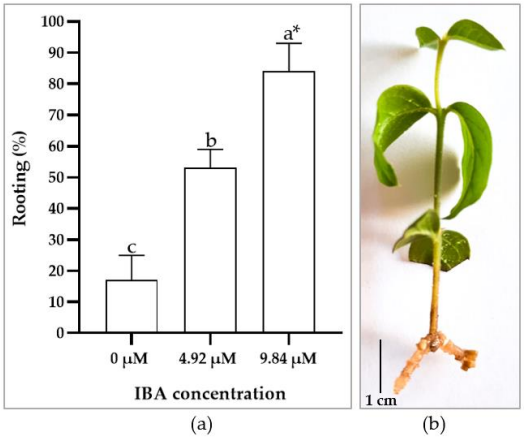

Figure 5. Response of *P. boldus* shoots on in vitro rooting stage. (**a**) Rooting rate (%); (**b**) Rooted explant (T3 Treatment) of *P. boldus*. T1: 0 (control); T2: 4.92 µM IBA; T3: 9.84 µM IBA. * The data in the bar (mean ± standard deviation) with different letters are significantly different. Tukey test ($p \leq 0.05$). Scale bar 1 cm.

Meanwhile, rooted vitroplants transferred to a cold greenhouse and transplanted into a substrate of perlite and peat in a 1:1 ratio achieved 100% survival at 30 days of growth (Figure 6).

Figure 6. Acclimated *P. boldus* plants.

4. Discussion

Our study has enabled the effective establishment of an in vitro propagation protocol for the species *Peumus boldus*, using different seed-derived mother plants. Nodal sections were utilized as the source of explants for the conducted experiments. This regeneration process centered around the proliferation of shoots from axillary buds, contributing to the mitigation of chromosomal variation [30]. Notably, our investigation achieved a survival rate of 74% during the initial in vitro establishment phase by utilizing the MS basal growth medium for the propagation of the four selected mother plants.

Similarly, notable successes have been observed in the micropropagation of various other woody species, such as *Cotoneaster* sp., *Betula lenta*, and *Citronella mucronata* [31–33], using the MS basal medium. It is important to emphasize that the success of propagation using a growth medium depends on several critical factors. These include the choice of explant, the physiological state of the mother plant, the type and concentration of sterilizing agents, and the duration of the sterilization process. It is worth noting that certain agents can adversely affect plant tissues, potentially leading to a reduction in the number of viable plants throughout the process [34]. The composition of the MS basal growth medium, which includes mineral salts (macro- and micronutrients), vitamins, and organic compounds, makes it well-suited for the in vitro propagation of a wide range of plant species [35].

In this study, the concentration of 4.44 µM BAP in the growth medium yielded an average shoot height of 5.9 cm and an average shoot count of 3.2. These outcomes are consistent with those observed in *Betula lenta*, where the optimal cytokinin concentration for effective shoot multiplication was found to be 5 µM BAP [32]. In contrast, for *Calophyllum brasiliense*, the application of BAP concentrations ranging from 4.4 to 8.8 µM resulted in an average of 4.4 and 4.6 shoots per nodal section after 90 days of cultivation [36]. Remarkably, the growth of *Euonymus verrucosus* shoots exhibited a significant boost to 3.7 cm within just 4 weeks upon the supplementation of the growth medium with 4.4 µM BAP [37].

Another vital aspect to consider revolves around the utilization of growth regulators, specifically auxins. In the context of this study, our efforts resulted in an impressive 85% rooting rate by enriching the MS growth medium with 9.84 µM IBA. By way of comparison, the woody species *Acacia confusa* achieved a rooting rate of 72% with 4.92 µM IBA [38], while species within the *Castanea* spp. genus showed rooting rates ranging from 67% to 97% with 2.46 µM IBA [39]. Conversely, in *Vaccinium corymbosum*, the use of 2.46 µM and

4.92 µM IBA resulted in rooting rates as high as 84% and 100%, respectively [40]. On a different note, the species *Citronella mucronata* showed a rooting rate of 60% when exposed to 14.76 µM IBA [33].

Furthermore, the adoption of an alternative substrate in lieu of agar led to a positive enhancement in the rooting ability of *P. boldus* shoots. The substrate, composed of perlite and peat in a 1:1 ratio, facilitated an increase in both root count and length, resulting in a remarkable rooting success rate of 77%. The effectiveness of this substrate is linked to multiple factors, particularly its porosity, which critically influences the delicate balance between aeration and water retention [41].

In this study, we achieved a 100% survival rate at the 30-day mark during the acclimation phase of rooted *P. boldus* vitroplants. This success can be attributed to the use of a substrate composed of peat and perlite in a 1:1 ratio. Comparable outcomes, with an 83% survival rate, were reported in the acclimation phase of *Psidium guajava*, using a substrate mixture of perlite, peat, and vermiculite in a 1:1:1 ratio [42]. In contrast, for *Pyrus communis*, a substrate mixture of perlite and peat in a 1:3 ratio yielded a 90% survival rate [43]. Similarly, rooted *Pyracantha angustifolia* plants, after being transplanted to a substrate of perlite and peat featuring a 2:1 (v/v) ratio, demonstrated a 92% survival rate [44]. Remarkably, the results of this study closely mirror those of the woody species *Citronella mucronata*, where vitroplants achieved a full 100% survival rate at the 30-day mark in a greenhouse environment, utilizing a substrate composed of peat and perlite in a 1:1 (v/v) ratio [33].

Through micropropagation, this research obtained a multiplication rate of 4.5 shoots per initial shoot; on the other hand, 85% rooting and 100% survival of the vitroplants were achieved. In contrast to the natural way, this species has been propagated by cuttings and seeds with success rates of 16% and 44%, respectively [13,16,17].

5. Conclusions

This study successfully established a protocol for mass propagation of *Peumus boldus* using the MS basal growth medium for in vitro establishment. The addition of BAP enhanced shoot length, count, and proliferation rate, while the incorporation of IBA increased the percentage of rooted plants. During acclimation, a 100% survival rate was achieved.

This research highlights the effectiveness of micropropagation protocols for woody species native to Chile's sclerophyllous forests. Furthermore, this method offers potential benefits for propagation, conservation, and the sustainable multiplication of this valuable medicinal plant, aligning with Chile's commitments under the Paris Agreement.

The protocol developed in this study allowed 4.5 plants per initial shoot to be obtained, the cultivation period being between 8 and 9 months for this species.

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References

1. Grau, J.; Zizka, G. *Chiles Pflanzenwelt*; Palmengarten Sonderheft 19: Fráncfort del Meno, Germany, 1992; p. 42.
2. Montenegro, G. *Chile Nuestra Flora útil. Guía Para Uso Apícola, Medicinal, Folclórica, Artesanal y Ornamental*; Universidad Católica de Chile: Santiago, Chile, 2000; p. 267.
3. Doll, U.; Aedo, D.; Lopez, P. Caracterización morfológica de tres procedencias de boldo (*Peumus boldus*) en una plantación joven de 6 años. *Bosque* **2005**, *26*, 45–54. [CrossRef]
4. Hoffmann, A.; Farga, C.; Lastra, J.; Veghazi, E. *Plantas Medicinales de Uso Común en Chile*, 3rd ed.; Fundación Claudio Gray: Santiago, Chile, 2003; p. 275.
5. Barros, S.; Benedetti, S. *Boldo (*Peumus boldus *Mol.) Rescate de Un Patrimonio Forestal Chileno. Manejo Sustentable y Valorización de Sus Productos*; Instituto Forestal: Santiago, Chile, 2011; p. 235.
6. Salehi, B.; Sharifi-Rad, J.; Herrera-Bravo, J.; Salazar, L.; Delporte, C.; Barra, G.; Cazar, M.E.; López, M.; Ramírez-Alarcón, K.; Cruz-Martins, N.; et al. Ethnopharmacology, phytochemistry and biological activities of native chilean plants. *Curr. Pharm. Des.* **2020**, *27*, 953–970.
7. Speisky, H.; Cassels, B. Boldo and boldine: An emerging case of natural drug development. *Pharmacol. Res.* **1994**, *29*, 1–12.
8. Kubinova, R.; Machala, M.; Minksova, K.; Neca, J.; Suchý, V. Chemoprotective activity of boldine: Modulation of drug-metabolizing enzymes. *Pharmazie* **2001**, *56*, 242–243.
9. O'Brien, P.; Carrasco-Pozo, C.; Speisky, H. Boldine and its antioxidant or health-promoting properties. *Chem. Biol. Interact.* **2006**, *159*, 1–17.
10. Verdeguer, M.; García-Rellán, D.; Boira, h.; Pérez, E.; Gandolfo, S.; Blázquez, M. Herbicidal activity of *Peumus boldus* and *Drimys winteri* essential oils from Chile. *Molecules* **2011**, *16*, 403–411. [CrossRef]
11. Uquiche, E.; Huerta, E.; Sandoval, A.; Del Valle, J.M. Effect of boldo (*Peumus boldus* M.) pretreatment on kinetics of supercritical CO_2 extraction of essential oil. *J. Food Eng.* **2012**, *109*, 230–237.
12. Soto, C.; Caballero, E.; Pérez, E.; Zúñiga, M.E. Effect of extraction conditions on total phenolic content and antioxidant capacity of pretreated wild *Peumus boldus* leaves from Chile. *Food Bioprod. Process.* **2014**, *92*, 328–333. [CrossRef]
13. Vogel, H.; Rasmilic, I.; San Martín, J.; Doll, U.; González, B. *Plantas Medicinales Chilenas. Experiencias de Domesticación y Cultivo de Boldo, Matico, Bailahuén, Canelo, Peumo y Maqui*; Universidad de Talca: Talca, Chile, 2005; p. 194.
14. Lara, A.; Little, C.; Urrutia, R.; Mcphee, J.; Alvarez-Garretón, C.; Oyarzún, C.; Soto, D.; Donoso, P.; Nahuelhual, L.; Pino, M.; et al. Assessment of ecosystem services as an opportunity for the conservation and management of native forests in Chile. *For. Ecol. Manag.* **2009**, *258*, 415–424.
15. Castañeda, L.E.; Godoy, K.; Manzano, M.; Marquet, P.A.; Barbosa, O. Comparison of soil microbial communities inhabiting vineyards and native sclerophyllous forests in central Chile. *Ecol. Evol.* **2015**, *5*, 3857–3868. [CrossRef] [PubMed]
16. Santelices, R.; Bobadilla, S. Arraigamiento de estacas de *Quillaja saponaria* Mol. y *Peumus boldus* Mol. *Rev. Bosque* **1997**, *18*, 77–85. [CrossRef]
17. Figueroa, J.; Jaksic, F. Latencia y banco de semillas en plantas de la región mediterránea de Chile central. *Rev. Chil. Hist. Nat.* **2004**, *77*, 201–215. [CrossRef]
18. Ríos, D.; Sandoval, D.; Gómez, D. In vitro culture of *Peumus boldus* Molina via direct organogenesis. *J. Med. Chem.* **2010**, *2*, 70–72.
19. Koch, Z.; González, J.; Benedetti, S. Regeneración de plantas in vitro de *Peumus boldus* Mol. (Boldo) mediante organogénesis de brotes epicórmicos de árboles maduros. *Cienc. Investig. For.* **2018**, *24*, 57–74. [CrossRef]
20. Machakova, I.; Zazimalova, E.; George, E. Plant growth regulators I. Introduction: Auxins, their analogues and inhibitors. In *Plant Propagation by Tissue Culture*; George, E.F., Hall, M.A., Klerk, G.J.D., Eds.; Springer: Dordrecht, The Netherlands, 2008; pp. 175–204.
21. Brault, M.; Maldiney, R. Mechanisms of cytokinin action. *Plant Physiol. Biochem.* **1999**, *37*, 403–412. [CrossRef]
22. Kumar, S.; Singh, R.; Kalia, S.; Sharma, S.; Kalia, R. Recent advances in understanding the role of growth regulators in plant growth and development in vitro-I: Conventional growth regulators. *Indian For.* **2016**, *142*, 459–470.
23. Saxena, A.; Shukla, M.; Saxena, P. Synthetic Seeds: Relevance to Endangered Germplasm Conservation In Vitro. In *Synthetic Seeds*; Springer: Cham, Switzerland, 2019; pp. 21–60.
24. Bi, W.; Saxena, A.; Ayyanath, M.-M.; Harpur, C.; Shukla, M.R.; Saxena, P.K. conservation, propagation, and redistribution (CPR) of hill's thistle: Paradigm for plant species at risk. *Plant Cell Tissue Organ Cult.* **2021**, *145*, 75–88. [CrossRef]
25. Saxena, A.; Bi, W.L.; Shukla, M.R.; Cannings, S.; Bennett, B.; Saxena, P.K. Micropropagation and cryopreservation of Yukon Draba (*Draba yukonensis*), a Special Concern Plant Species Endemic to Yukon Territory, Canada. *Plants* **2021**, *10*, 2093. [CrossRef]
26. Santibáñez, F. *Atlas Agroclimático de Chile. Estado Actual y Tendencias del Clima. Tomo III: Regiones de Valparaíso, Metropolitana, O'Higgins y Maule*; Fundación para la Innovación Agraria: Santiago, Chile, 2017; p. 37.
27. Lloyd, G.; McCown, B. Commercially-feasible micro-propagation of Mountain laurel, Kalmia latifolia, by use of shoot tip culture. *Comb. Proc. Int. Plant Prop. Soc.* **1980**, *30*, 421–427.
28. Murashige, T.; Skoog, F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* **1962**, *15*, 473–497. [CrossRef]
29. Driver, J.A. In vitro Propagation of paradox walnut rootstock. *HortScience* **1984**, *19*, 507–709. [CrossRef]
30. Ostry, M.; Hackett, W.; Michler, C.; Serres, R.; McCown, B. Influence of regeneration method and tissue source on the frequency of somatic variation in Populus to infection by *Septoria musiva*. *Plant Sci.* **1994**, *97*, 209–215. [CrossRef]

31. Monier, C.; Ochatt, S.J. Establishing micropropagation conditions for five *Cotoneaster* genotypes. *Plant Cell Tissue Organ Cult.* **1995**, *42*, 275–281. [CrossRef]
32. Rathwell, R.; Shukla, M.R.; Jones, A.M.P.; Saxena, P.K. In vitro propagation of cherry birch (*Betula lenta* L.). *Can. J. Plant Sci.* **2016**, *96*, 571–578. [CrossRef]
33. Guerra, F.; Badilla, L.; Cautin, R.; Castro, M. Micropropagation of *Citronella mucronata* D. Don, a Vulnerable Chilean Endemic Tree Species. *Plants* **2022**, *11*, 2425. [CrossRef]
34. Silva, J.; Winarto, B.; Dobránszki, J.; Zeng, S. Disinfection procedures for in vitro propagation of Anthurium. *Folia Hortic.* **2015**, *27*, 3–14. [CrossRef]
35. Khatri, P.; Rana, J.S.; Sindhu, A.; Jamdagni, P. Effect of additives on enhanced in-vitro shoot multiplication and their functional group identification of *Chlorophytum borivilianum* Sant. Et Fernand. *SN Appl. Sci.* **2019**, *1*, 1105. [CrossRef]
36. Silveira, S.S.; Cordeiro-Silva, R.; Degenhardt-Goldbach, J.; Quoirin, M. Micropropagation of *Calophyllum brasiliense* (Cambess.) from nodal segments. *Braz. J. Biol.* **2016**, *76*, 656–663. [CrossRef]
37. Kirillov, V.; Pathak, A.; Stikhareva, T.; Ercisli, S.; Daulenova, M.; Kazangapova, N.; Rakhimzhanov, A. In vitro propagation and ex vitro rooting of *Euonymus verrucosus* Scop. (Celastraceae)—A rare species of Kazakhstan flora on the southern border of its areal. *J. For. Res.* **2022**, *27*, 289–296. [CrossRef]
38. Ho, W.J.; Huang, Y.K.; Huang, W.W. Effective in vitro culture using dormant bud of nodal sections from a mature *Acacia* tree. *In Vitro Cell. Dev. Biol.-Plant* **2022**, *58*, 437–446. [CrossRef]
39. Mao, A.A.; Vijayan, D.; Singha, R.K.N.; Pradhan, S. In vitro propagation of *Rhododendron wattii* Cowan—A critically endangered and endemic plant from India. *In Vitr. Cell. Dev. Biol.-Plant* **2018**, *54*, 45–53. [CrossRef]
40. Guo, Y.X.; Zhao, Y.Y.; Zhang, M.; Zhang, L.Y. Development of a novel in vitro rooting culture system for the micropropagation of highbush blueberry (*Vaccinium corymbosum*) seedlings. *Plant Cell Tissue Organ Cult.* **2019**, *139*, 615–620. [CrossRef]
41. Damiani, C.R.; Schuch, M.W. Diferentes substratos e ambientes no enraizamento in vitro de mirtilo. *Cienc. Rural* **2009**, *39*, 563–566. [CrossRef]
42. Tzatzani, T.T.; Michail, I.; Boutsika, A.; Sarrou, E.; Ganopoulos, I. Micropropagation of guava (*Psidium guajava*) seedlings, a plant with interest in cool subtropics, using an innovative BB culture medium. *Biotechnol. Biotechnol. Equip.* **2023**, *37*, 139–150. [CrossRef]
43. Kaviani, B.; Barandan, A.; Tymoszuk, A.; Kulus, D. Optimization of In Vitro Propagation of Pear (*Pyrus communis* L.) 'Pyrodwarf®(S)' Rootstock. *Agronomy* **2023**, *13*, 268. [CrossRef]
44. Kaviani, B.; Deltalab, B.; Kulus, D.; Tymoszuk, A.; Bagheri, H.; Azarinejad, T. In Vitro Propagation of *Pyracantha angustifolia* (Franch.) C.K. Schneid. *Horticulturae* **2022**, *8*, 964. [CrossRef]