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RESEARCH PAPER

In vitro propagation of *Gaultheria pumila* (L.f.) D.J. Middleton (Ericaceae), a Chilean native berry with commercial potential

José Pico-Mendoza[1], Rolando Garcia-Gonzáles[2], Karla Quiroz[4], Borys Chong[2], Hugo Pino[4], and Basilio Carrasco[3]

[1]Universidad Técnica de Manabí, Facultad de Ingeniería Agronómica, Carrera de Agronomía. Lodana-Manabí- Ecuador.

[2]Research and Services Society, BioTECNOS Ltda. 49 ½ East, 2385, Parque del Sol, Talca, Chile.

[3]Centro de Estudios en Alimentos Procesados (CEAP). Av. Lircay s/n Box 1007. Talca. Chile.

[4] Catholic University of Maule, Department of Forest Sciences, Natural Resources Biotechnology Center. San Miguel Avenue 3605, box 617, Talca, Chile.

Abstract

J. Pico-Mendoza, R. Garcia-Gonzáles, K. Quiroz, B. Chong, H. Pino, and B. Carrasco. 2021. *In vitro* propagation of *Gaultheria pumila* (L.f.) D.J. Middleton (Ericaceae), a Chilean native berry with commercial potential. Int. J. Agric. Nat. Resour. 83-96. A micropropagation protocol for *G. pumila* was developed. Young shoots were collected during the growing season (October to December 2016) from a wild population in the Villarrica Volcano area in the Araucanía Region of Chile. Nodal segments were used for *in vitro* initiation after testing several disinfection treatments with different concentrations of sodium hypochlorite. Disinfected explants were placed onto 100% WPM basal medium (WPM100) supplemented with a range of concentrations of 2-iP (2-isopentenyladenine) to evaluate the best regeneration media during *in vitro* culture. Disinfection with 1% sodium hypochlorite for 40 minutes, followed by a second disinfection with 2% sodium hypochlorite for 25 minutes, and cultivation on MS basal medium supplemented with 2 mg L^{-1} 2-iP gave the highest efficiency of disinfected plants. In the propagation stage, the highest multiplication rates were obtained when 1 mg L^{-1} zeatin was added to the basal WPM100 medium. *In vitro* rooting and preacclimation were better when elongated plants were cultivated on WPM100 supplemented with 3 mg L^{-1} naphthalene acetic acid. This *in vitro* protocol could be used to propagate genotypes of this Chilean native species and is also an important tool toward its domestication and commercial use.

Keywords: Ericaceae, *Gaultheria* sp., *in vitro* rooting, micropropagation, plant growth regulators.

Introduction

Gaultheria pumila (L.f.) D.J. Middleton (Ericaceae) is a wild berry species native to Chile. *G. pumila* is a low bush (up to 80 cm height), and it is commonly known as *chaura* or *mutilla del zorro* due to its fleshy fruits that are eaten by small mammals and other animals. In addition, *G. pumila* has several characteristics, such as high diversity of fruits, morphometric traits, adaptability to several ecological conditions and high content of polyphenols (Middleton, 1992; Villagra *et al.,* 2014). All these properties make this species very

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Corresponding author: newthon.pico@utm.edu.ec

attractive for cultivation as a commercial plant, particularly taking advantage of its nutritional potential as a functional food (Lasekan, 2014) as well as its ecological plasticity. Currently, specific biotechnological tools, such as SSR markers have been developed (Garcia-Gonzales *et al.,* 2018) for genetic diversity studies (Pico-Mendoza *et al.,* 2020).

Similar to many other Chilean native fruits, *G. pumila* has a rich and diversified composition of bioactive compounds with potential health benefits (Schreckinger *et al.,* 2010; Ruiz *et al.,* 2013). *G. pumila* has been used as a food and medicinal plant by indigenous people in Chile, such as the Aónikenk, Selk'nam, Kawésqar, Yagan, and Haush people from southern Chilean Patagonia (Dominguez, 2010). Several compounds have been identified, such as polyphenols (Middleton, 1992), anthocyanins (Villagra *et al.,* 2014) essential oils (Bantawa *et al*., 2011), and high contents of methyl salicylate-rich essential oils (Apte *et al.,* 2006). They have pharmacological characteristics such as anti-inflammatory, antioxidant, antibacterial, analgesic (Liu *et al.,* 2013), and potential anticancer activities (Luo *et al.,* 2018).

G. pumila is mainly reproduced by seeds in the wild. The seeds are small, and each fruit contains approximately 50 seeds per fruit or more. The species can also be propagated asexually by underground stems forming shoots during the warmer seasons, producing new clusters of plants around the original mother plant.

No information has been generated for the asexual propagation of *G. pumila*. However, there are publications for the Indian Wintergreen (*Gaultheria fragrantissima* Wall.), which is normally propagated by seeds or via rooted cuttings, but both methods are very slow (Ranyaphi *et al.,* 2012). Micropropagation through shoots was reported for *G. fragrantissima* (Bantawa *et al.,* 2011; Ranyaphi *et al.,* 2012). Media used for *in vitro* culture include woody plant medium (WPM) supplemented with different concentrations of thidiazuron (TDZ) alone or in combination with 6 benzyl-amino purine (BAP); kinetin (Kin) alone or in combination with auxin indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), or α-naphthalene acetic acid (NAA) (Bantawa *et al*., 2011) and semisolid rhododendron medium (RM), Murashige and Skoog medium (MSM) and WPM supplemented with N-6-benzyladenin (BA), kinetin (Kin) and 2-iso-pentenyladenine (2iP) (Ranyaphi *et al.,* 2012).

In vitro culture offers other advantages, such as developing disease-free plants of the species under study and rapidly scaling up for commercial production (García-Gonzáles *et al*., 2010). Considering the efficiency of *in vitro* propagation techniques, their application in *G. pumila* would ideally provide a fast and efficient propagation system while maintaining high health status. One of the more relevant applications of tissue culture in plants is the support of domestication projects of noncultivated plants (Gepts, 2004a, 2004b) since it is necessary to produce enough plants from selected genotypes in a limited period of time to evaluate the agronomic performance under cultivated conditions. This study aimed to develop the first micropropagation protocol for *G. pumila*, addressing the different steps of the tissue culture process.

Materials and methods

Plant material

Plants of *G. pumila* were selected in actively growing areas at Villarrica National Park, Araucanía Region (S: 39°34'463, W: 71°28'509). The plants were transported to the greenhouse with soil in a humid cooler box. Once in the greenhouse, the plants were planted in five liter pots containing soil brought from the sampling location. These mother plants were watered daily with 200 ml of water. No fertilizer or pesticides were used during the preparation of the plants. Light pruning was performed to eliminate older branches as well as

dead leaves to stimulate shoot formation. After new shoot production, healthy 10 cm explants were collected and prepared in 1.0 cm nodal pieces containing two to three vegetative buds.

General environmental conditions

For all experiments in this study, the basal media was supplemented with 3% (w/v) sucrose and 8% (w/v) agar. The pH of the media was adjusted to 5.7 before sterilization by autoclaving for 20 minutes at 1 kg cm^{-2} and 121 °C. For all the experiments described above, the cultures were transferred into a culture room at 24 ± 1 °C and a 12/12 photoperiod under cool white fluorescent lamps (60 µmol m^{-2} s^{-1}).

Disinfection and in vitro *initiation*

Nodal explants of 1.0 cm containing two to three vegetative buds were used. The selected explants were washed using distilled water and common detergent for twenty minutes, followed by a deep wash using sterile distilled water (500 ml) with three drops of Tween 20 for 20 min, and then rinsed three times with sterile distilled water to eliminate any detergent residues. These steps were performed under a laminar flow chamber. Once rinsed, the disinfection treatments were applied to the nodal explants. After disinfection, the explants were rinsed three times with sterile distilled water. Oxidized tissues were removed from the explants before planting them on semisolid Murashige and Skoog (MS) medium (Murashige & Skoog 1962) supplemented with three concentrations of 2-iP: 0.5, 1.0, and 2.0 mg L^{-1}. The final evaluation of the experiment was performed five weeks after initiation. For the disinfection procedure, six treatments were tested (Table 1). Each treatment had twenty-one replicates planted in a single jar. The effects of each treatment on the number of contaminated explants and the number of regenerated plants were evaluated to choose the best disinfection protocol.

Plant formation and stabilization of in vitro *plantlets*

After selecting the best disinfection and *in vitro* initiation conditions, it was necessary to stabilize the development of the established explants. The effect of the type of cytokinin (2 isopentenyl ad-

Table 1. Treatments applied for disinfection and *in vitro* initiation of *G. pumila* explants

Treatments	Sodiun hypochlorite concentration (%)	Desinfection time (minutes)	Murashige and Skoog (MS) medium and 2-iP (mg L^{-1})
T1	1	40	0.5
	2	25	
T2	1	40	1
	2	25	
T3	1	40	2
	2	25	
T4	1	30	0.5
	2	25	
T5	1	30	1
	2	25	
T6	1	30	2
	2	25	

enine, 2-iP; 6-bencil aminopurine, BAP; Zeatin, Zea) and their concentration (0.5, 1.0, and 2.0 mg L^{-1}) on the morphogenic response of established explants was evaluated. Each growth regulator at its test concentration was added to four different basal media: full-strength MS (MS100), half-strength MS medium (MS50), full-strength WPM (WPM100), and half-strength WPM medium (WPM50). Only explants coming from the selected disinfection and *in vitro* establishment protocol were used in this experiment. The medium preparation and sterilization were developed as described above. To successfully establish the tissue cultures, the effects of the basal medium, cytokinin type, and concentration on plantlet recovery were studied. Twenty-four multifactorial treatments were assayed with fifteen repetitions (Table 2). To select the best treatment, morphogenic responses were evaluated, such as the percentage of explants, the number of shoots, shoot length per explant, and the number of leaves.

Table 2. Treatments for the *in vitro* initiation of *G. pumila* in different media and the use of different plant growth regulators

Treatments	Basal Medium	Dilution of basal medium (%)	Growth regulator	Plant growth regulator concentration (mg L^{-1})
T1	MS	100	2-iP	1.0
T2	MS	100	2-iP	2.0
T3	MS	100	BAP	1.0
T4	MS	100	BAP	2.0
T5	MS	100	Zeatin	0.5
T6	MS	100	Zeatin	1.0
T7	MS	50	2-iP	1.0
T8	MS	50	2-iP	2.0
T9	MS	50	BAP	1.0
T10	MS	50	BAP	2.0
T11	MS	50	Zeatin	0.5
T12	MS	50	Zeatin	1.0
T13	WP	100	2-iP	1.0
T14	WP	100	2-iP	2.0
T15	WP	100	BAP	1.0
T16	WP	100	BAP	2.0
T17	WP	100	Zeatin	0.5
T18	WP	100	Zeatin	1.0
T19	WP	50	2-iP	1.0
T20	WP	50	2-iP	2.0
T21	WP	50	BAP	1.0
T22	WP	50	BAP	2.0
T23	WP	50	Zeatin	0.5
T24	WP	50	Zeatin	1.0

Effect of plant growth regulator interaction on the in vitro multiplication of G. pumila

For the multiplication step, all *in vitro* plants were grown in the best medium of the previous phase for two cycles of four weeks each. The plants selected were prepared as nodal explants harboring at least one bud. The explants were then planted on WPM100 medium with different concentrations of IBA combined with zeatin or 2-iP. Twelve treatments were replicated four times combining the interaction between auxins and cytokinin and tested to determine the best multiplication rate (Table 3). The effect of the different treatments was considered to choose the best micropropagation condition. After six weeks of culture, the effect of the different treatments on shoot number, shoot length, explant contamination and oxidation rate were evaluated.

Table 3. Treatments used for the *in vitro* multiplication of *G. pumila* with different concentrations and combinations of plant growth regulators.

IBA	Zeatin		2 -iP	
	1.0 mg L^{-1}	2.0 mg L^{-1}	2.0 mg L^{-1}	3.0 mg L^{-1}
0	T1	T2	T7	T8
0.25 mg L^{-1}	T3	T4	T9	T10
0.5 mg L^{-1}	T5	T6	T11	T12

Evaluation of auxins on the in vitro rooting

In vitro rooting and preparation of individual plantlets is critical in obtaining plantlet survival during the *ex vitro* phase. The effects of auxins, such as indolbutiric acid (IBA), indol-3-acetic acid (IAA), and naftalenacetic acid (NAA), added at different concentrations (1, 2, 3, and 4 mg L^{-1}) to the basal WPM medium on rooting and plant development were tested. Twelve treatments and thirty replicates were evaluated. The percentages of roots, roots per explant, and callus formation were evaluated to select the best *in vitro* rooting conditions.

Ex vitro *acclimation of individual plantlets*

For the *ex vitro* acclimation stage, several factors were considered, such as light intensity, substrate quality, and relative humidity in the adaptation tunnel. The variable measured for this stage was the number of surviving active plants. The substrate used had a mixture of 50% organic matter, 25% vermiculite, and 25% sand. The explants came from the previous rooting phase, using ten explants for each treatment. Before being planted, the plantlets were immersed in a 1 g L^{-1} IBA solution for two minutes. The temperature conditions in the greenhouse were 5 °C low 20 °C high during the winter months, and 15 °C low and 35 °C high during the summer months. The humidity inside the greenhouse ranged between 55–60% year-round.

Statistical analysis

Random block design (RBD) was used for all the experiments. Statistical analyses were performed using InfoStat statistical software (Balzarini *et al*., 2008). The normality of the data was determined by the Q-Q plot graphic and corroborated with the Shapiro–Wilks test of residues. To detect the best treatment, an analysis of variance (ANOVA) and Tukey HSD test with a 95% confidence level were performed if the data had a normal distribution of variance. For those not complying with the normality assumptions, a Kruskal–Wallis test with a level of significance of 95% was performed.

Results

Effect of the disinfection protocol and plant growth regulators on explant decontamination and plantlet development during in vitro establishment

Disinfection and initiation

The highest disinfection efficiency ($p=0.01$) was obtained when the explants were exposed to treat-

ment T3, disinfection with 1% sodium hypochlorite for 40 minutes, followed by a second wash with 2% sodium hypochlorite for 25 minutes, and cultivation on MS medium. Using this treatment, only 5% of contaminated explants were observed (Fig. 1). Fungi and bacteria were the main contaminants in this stage. However, the highest morphogenic response was observed in treatment T2, where the 2-iP concentration was reduced to 1 mg L^{-1} compared to T3. This combination of plant growth regulators in the disinfection phase helped the explants to be stimulated and to have enough material for the next stages of micropropagation. Using this 2-iP concentration produced an increase in shoot production per explant of up to 56%, which was significantly different from the rest of the treatments ($p=<0.01$).

Plantlet formation and stabilization *in vitro*

The stabilization of the explants after disinfection was necessary to homogenize their morphogenic response. It was found that the cultivation of the disinfected explants in treatment 24 (WPM50 +

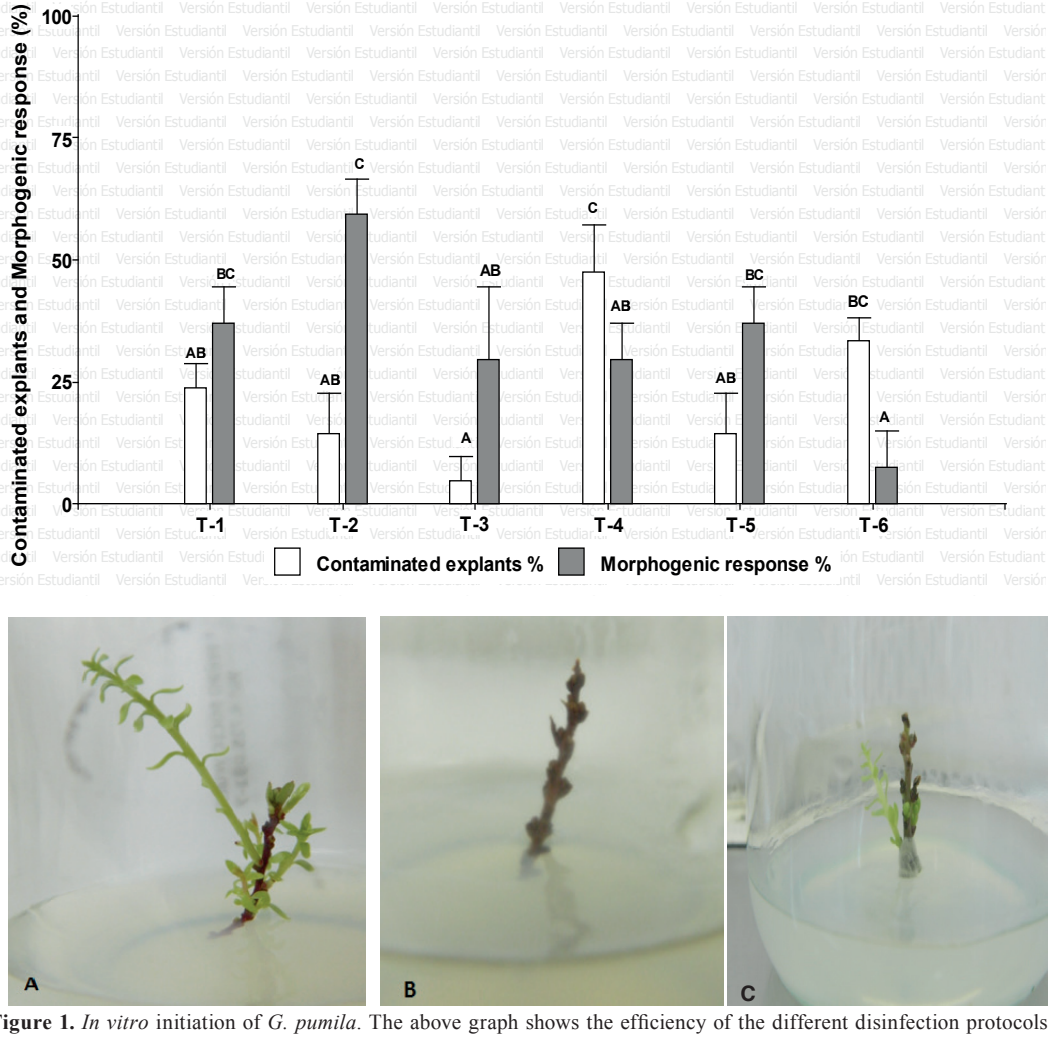

Figure 1. *In vitro* initiation of *G. pumila*. The above graph shows the efficiency of the different disinfection protocols tested during *in vitro* initiation. The percentage of contaminated explants and morphogenic response as percentage of shoot formation in each treatment is displayed. Below, behavior of explants during *in vitro* initiation; **A**: shoot formation from an aseptic explant; **B**: oxidized explant seven days after disinfection and *in vitro* culture onto regeneration medium; **C**: Explant contaminated with fungi.

1.0 mg L^{-1} Zeatin) produced more shoots than the rest of the treatments. In second place was treatment 18 (WPM100 + 1.0 mg L^{-1} zeatin), followed by treatments 14 and 13. However, treatment 16 (WPM100% + BAP 2.0 mg L^{-1}) presented the longest shoots and the highest number of leaves. In general, the treatments that were supplemented with 2iP and zeatin showed the best-developed shoots (Table 4).

Effects of the interaction of auxin and cytokinins on the morphogenic response during in vitro multiplication

Micropropagation was significantly influenced by the different treatments tested ($p \leq 0.05$). When placed on treatment T1 (WPM100 + 1.0 mgL^{-1} Zeatin), an average of six shoots per explant was obtained with an average shoot length of 4.8 cm

Table 4. Effect of culture media and plant growth regulators on plant morphogenesis of *G. pumila*. Only treatments with the highest number of explants with shoot formation are shown. Values in the same column followed by the same letter do not differ significantly according to the Kruskal–Wallis test (p≤0.05).

Treatments	Shoot formation (number)	Shoot formation (%)	Shoots formation per explant	Average of shoot length (mm)	Number of leaves/shoots
T13 (WPM100%+2-iP 1.0 mg L-1)	2	4	2.0±1.0a	7.5±6.3b	4.5±6.3b
T14 (WPM100%+2-iP 2.0 mg L-1)	7	16	2.1±1.4a	13.8±4.4b	6.7±1.9ab
T16 (WPM100%+BAP 2.0 mg L^{-1})	3	7	1.0±0.0a	24.3±10a	11.0±6.5a
T18 (WPM100%+Zeatin 1.0 mg L^{-1})	21	47	2.1±1.0a	8.3±2.9b	4.2±2.2b
T23 (WPM50%+Zeatin 0.5 mg L^{-1})	7	16	1.1±0.3a	9.7±3.3b	1.7±1.7b
T24 (WPM50%+Zeatin 1.0 mg L^{-1})	13	29	2.5±1.0a	11.5±3.9b	6.0±2.6ab
p = value			>0.05	<0.01	<0.01

Table 5. *In vitro* morphogenic response of *G. pumila* using different culture media and plant growth regulators. Values in the same column followed by the letter do not differ significantly according to Tukey's HSD test (p≤0.05).

Treatments	Average shoot length (cm)	Shoots formation per explant (number)
T1(WPM100+1 mgL^{-1} Zeatin)	4.8±1.0 a	6.0±0.8 a
T2(WPM100+2 mgL^{-1} Zeatin)	3.3±1.0 b	4.3±0.5 b
T3(WPM100+1 mgL^{-1} Zeatin +0.25 mg L^{-1} IBA)	2.3±0.5 b	3.3±0.5 c
T4(WPM100+2 mgL^{-1} Zeatin +0.25 mg L^{-1} IBA	1.5±0.6 c	2.8±1.0 c
T5(WPM100+1 mgL^{-1} Zeatin +0.5 mg L^{-1} IBA)	2.5±0.6 b	3.0±0.0 c
T6(WPM100+2 mgL^{-1} Zeatin +0.5 mg L^{-1} IBA)	2.5±0.6 b	3.5±1.3 c
T7(WPM100+2 mgL^{-1} 2-iP)	3.0±0.8 b	4.5±0.6 b
T8(WPM100+3 mgL^{-1} 2-iP)	2.8±0.6 b	4.8±1.0 b
T9(WPM100+2 mgL^{-1} 2-iP + 0.25 mgL^{-1} IBA)	1.3±1.0 c	3.3±0.5 c
T10(WPM100+3 mgL^{-1} 2-iP + 0.25 mgL^{-1} IBA)	1.0±0.0 c	2.8±0.5 c
T11(WPM100+2 mgL^{-1} 2-iP +0.5 mgL^{-1} IBA)	0.8±0.5 c	2.5±0.6 c
T12(WPM100+3 mgL^{-1} 2-iP +0.5 mgL^{-1} IBA)	0.8±0.5 c	2.8±0.5 c
p value	<0.01	<0.01

(Table 5). Treatment 2 (WPM100 2.0 mgL^{-1} Zeatin) produced an average shoot length of 3.3 cm per explant, with 4.3 shoots per explant. After six weeks of culture, the regenerated shoots showed expanded leaves and grew vigorously (Fig. 2). Oxidation of the explants was very low in all the experiments, with no significant differences between treatments ($p \geq 0.05$).

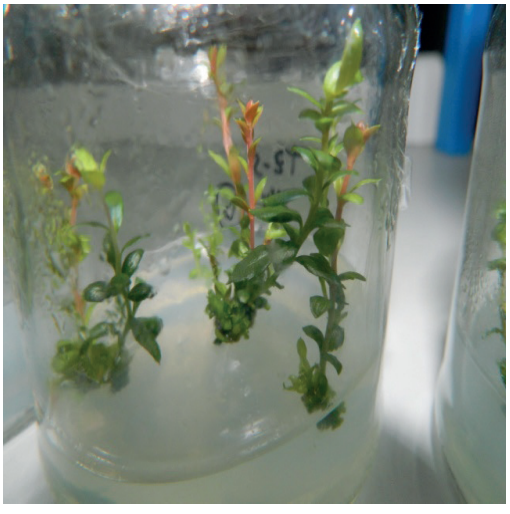

Figure 2. Response of *in vitro* nodal segments to different concentrations and combinations of plant growth regulators in WPM100 medium in the multiplication stage in *G. pumila*.

Evaluation of auxins on the in vitro rooting and preacclimatization of individualized plantlets

The addition of auxins to the basal medium significantly increased the efficiency of root formation. At the same time, the addition of 3 mg L^{-1} NAA induced the highest rooting efficiency (47% rooted explants, $p=0.01$), followed by the addition of 4 mg L^{-1} NAA, which produced 40% explant rooting, with means of 1.23 and 1.13 roots per explant in treatments T11 (WPM + 3 mg L^{-1} NAA) and T12 (WPM + 4 mg L^{-1} NAA), respectively. However, treatments T1, T2, T3, T4, T5, T6, and T8, despite having been supplemented with auxins, did not produce roots (Table 6). Callus formation was induced more efficiently when media were supplemented with NAA and IBA.

Ex vitro *acclimation*

Of the explants exposed to *ex vitro* conditions, only 50% survived when moved to greenhouse conditions. The explants that were planted showed a good root system after 45 days of sowing (Fig. 3).

Discussion

Any explants to be introduced *in vitro* need a surface disinfection process that has to be nondamaging to the plant tissue but efficient in controlling microorganisms (García-Gonzáles *et al*., 2010). This phase is paramount and one of the most important phases for the establishment of the species. Surface cleaning of explants repeated prior to disinfection decreases the presence of contaminants, and this decrease is greater when explants are sterilized more than twice. Sodium hypochlorite, calcium hypochlorite, ethanol, and mercuric chloride are the most common disinfectants used during this initial step (Garcia *et al*., 1999; Husain & Anis, 2009; Singh & Gurung, 2009; Tilkat *et al*., 2009).

Table 6. *In vitro* root formation in *G. pumila* plants induced by different media and plant growth regulators. nr: no response. Different letters in the same column indicate significant differences between treatments. Kruskal–Wallis test ($p \leq 0.05$).

Treatments	Percentage of roots	Roots per explant	Calli formation (means+SE)
T1(WPM +1 mgL^{-1} IBA)	nr	nr	0.40±0.50bc
T2 (WPM +2 mgL^{-1} IBA)	nr	nr	0.17±0.38c
T3(WPM +3 mgL^{-1} IBA)	nr	nr	0.47±0.51b
T4(WPM +4 mgL^{-1} IBA)	nr	nr	0.40±0.50bc
T5(WPM +1 mgL^{-1} IAA)	nr	nr	nr
T6(WPM +2 mgL^{-1} IAA)	nr	nr	nr
T7(WPM +3 mgL^{-1} IAA)	3	0.03 ±0.18b	nr
T8(WPM +4 mgL^{-1} IAA)	nr	nr	nr
T9(WPM +1 mgL^{-1} NAA)	23	0.43± 0.9ab	0.83±0.38a
T10(WPM +2 mgL^{-1}NAA)	27	1.0±1.86ab	0.93±0.25a
T11(WPM +3 mgL^{-1} NAA)	47	1.23±1.5a	0.97±0.18a
T12(WPM +4 mgL^{-1} NAA)	40	1.13±1.96a	1.00±0.00a

Figure 3. *Ex vitro* acclimatation of *G. pumila* plants under greenhouse conditions. A: *G. pumila* explants after the *in vitro* rooting stage. B: Functional roots after 20 days of acclimation. C: Explants of *G. pumila* established in the greenhouse prior to being transplanted to individual containers.

In this study, two cycles of disinfection (1% sodium hypochlorite for 40 minutes and 2% sodium hypochlorite for 25 minutes) and establishment in MS medium with low concentrations of salts were performed. Ninety five percent explant disinfection gave the best results for *in vitro* disinfection and morphogenesis during the establishment stage. Shoot formation and elongation were improved under this protocol of disinfection. Studies on *in vitro* micropropagation carried out in *Rhododendron ponticum L* (Almeida *et al*., 2005) using commercial bleach 25% (v/v) (5% sodium hypochlorite) for 20 minutes reported 43% contaminated explants; in other words, these explants still showed a high rate of contamination compared with our results. The combination of sterilization products is often

used with the intention of increasing asepsis in explants, especially in the Ericaceae family. In *Vaccinium arctostaphylos* L. and *Vaccinium myrtillus* L., surface sterilization with 70% ethanol for 1 minute followed by disinfection for 15 min with 3% sodium hypochlorite showed positive disinfection results (Cüce *et al*., 2013; Cüce & Sökmen, 2015).

In *Rhododendron ledebourii,* explants were submerged in 70% ethanol for 30 seconds, and then their surface was sterilized for 15 min by 0.15% (w/v) $HgCl_2$ with a drop of Tween-80. Using that method, 40% and 20% survival was obtained in two genotypes after surface sterilization (Erst *et al*., 2014). Other methods of sterilization have been used for the *in vitro* propagation of other berry species, such as strawberry (*Fragaria vesca L.*) (96% ethanol for 30 sec and 0.1% $HgCl_2$ for 3 min), raspberry (*Rubus idaeus* L.), (70% ethanol for 60 sec and 0.1% $HgCl_2$ for 5 min), bilberry (*Vaccinium myrtillus* L.) and lingonberry (*Vaccinium vitis-idaea* L.) (70% ethanol for 30 sec and 0.1% $HgCl_2$ for 3 min), with results showing over 80% efficiency in decontamination, with the exception of bilberry (Georgieva *et al*., 2016). All these results for different species and sterilization protocols are below the levels reported in this study. The difference in results is probably because we used a two-phase sterilization process, with a lower concentration of sodium hypochlorite, but increased the time that we treated the explants, which may be the key to success in the sterilization of explants for this species.

Likewise, with the intention of verifying the effect of the culture medium, different concentrations and types of cytokinins on the growth of *in vitro* plantlets of *G. pumila* were studied. In this case, the best results were obtained using WPM100 medium supplemented with zeatin at 1.0 mg L^{-1}, which produced 47% explants with shoots, showing the best homogeneity of explants, both in number and height. In *Vaccinium arctostaphylos,* WPM supplemented with 1.0 mg L^{-1} zeatin and 0.1 mg L^{-1} IBA appeared to be the best basal medium in terms of shoot formation, where 74% of the explants developed shoots, where the combination of WPM and zeatin showed better homogeneity in explant formation (Cüce *et al*., 2013) than that in *G. pumila.* The application of zeatin showed that a concentration of 1.0 mg L^{-1} promotes the induction and stabilization of shoots. Zeatin very effectively stimulates shoot multiplication, leaf formation, and the development of shoot length, as has happened in species of the *Vaccinium* genus (Gajdosová *et al.,* 2006; Cüce *et al*., 2013). In the multiplication stage, the addition of cytokinin to the WPM100 medium produced higher axillary propagation rates and shoot elongation, which were also significantly higher in these conditions.

On the other hand, supplementing the media with auxins did not have any significant effect on plant morphogenesis during the multiplication stage. Despite the importance of the interaction between auxin and cytokinin in the formation and maintenance of meristems for establishing the whole plant body (Su *et al*., 2011) in *G. pumila* that was not significant, this treatment showed the lowest value on multiplication rate. Only the effect of the cytokinin in the *G. pumila* explants showed a higher number of shoots and elongation. These results differ from the results obtained in *Fragaria chiloensis*, where the interaction of auxins and cytokinins is an important factor in the formation and improvement of new shoots and the number of leaves per shoot, even in the induction of roots in other species (Quiroz, 2014; Aremu *et al*., 2016). Regarding other studies in the Ericaceae family, the use of 2-iP in *Vaccinium macrocarpon* during the multiplication of the species induced longer shoots (approximately 4 cm) in different clones (Debnath & McRae, 2001). Additionally, it has been demonstrated that the morphogenic response to cytokines could depend strongly on genotype, as has been shown in *Arbutus unedo* L. (Gomes *et al*., 2010) and raspberry (Gajdošová *et al*., 2006).

In the *in vitro* root formation stage, the explants showed a low response in terms of root formation. Root induction was greater in treatments

that were supplemented with naphthalene acetic acid (NAA), where 47% of explants produced roots. In contrast to these results, in related species such as *Vaccinium corymbosum* L. cv. 'Elliot', the addition of BAP produced more than 90% rooted explants (Vescan *et al*., 2012). In the case of blueberry *in vitro* plantlets, the effects of IBA at different concentrations produced over 75% rooted explants (Guang-Jie *et al*., 2008). At this stage, in addition to the production of roots, the induction of calli and shoots are also stimulated. In *Casuarina cunninghamiana* Miq., first, the callus is formed, and later, the adventitious roots are formed from the callus (Shen *et al*., 2010). The formation of new organs in the rooting stage allows the survival of the explant, giving opportunity for the formation of roots in the longer term, especially in species difficult to root, as in the Ericaceae family. Finally, as in many Ericaceae species, *in vitro* rooting in *G. pumila* was very hard to induce. The addition of auxins, in general, produced some roots, but the efficiency was still low (47%).

The acclimatization of the *in vitro* plants in this study was lower (50%) than that reported in *G. fragrantissima,* which varied between 70% (Bantawa *et al*., 2011) and 80% (Ranyaphi *et al*., 2012). This difference could be due to the type of substrate used, since the highest percentage of survival reported occurred using 100% sand (Ranyaphi *et al*., 2012), followed by a 9:1:1 (v/v/v) mixture of virgin soil (upper layer of black jungle soil collected from a deep forest area), sand and manure (Bantawa *et al*., 2011). In the current study, the substrate was a mixture of 50% organic matter, 25% vermiculite, and 25% sand. The substrate probably did not have a sufficient proportion of sand, which allows better aeration and better development of the root system (Abul Soad *et al*., 2012).

Conclusions

The use of a little-explored genetic resource with high potentialities, such as *G. pumila,* has become an option as a horticultural crop. The micropropagation of *G. pumila* will allow mass production of plants, especially in genotypes of interest with desirable characteristics, which allows us to advance in a selection and breeding process. The intention is to turn the species into an alternative for the production of nontraditional crops for the horticultural sector. The results of this research provide an efficient *in vitro* protocol that guarantees the multiplication of this species at different scales. This study contributes to laying the initial foundations for the domestication of *G. pumila* and, in a relatively short period, we could benefit from its attributes in a sustainable way.

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Resumen

J. Pico-Mendoza, R. Garcia-Gonzáles, K. Quiroz, B. Chong, H. Pino, y B. Carrasco. 2021. Propagación *in vitro* de *Gaultheria pumila* (L.f.) D.J. Middleton (Ericaceae), una baya nativa chilena con potencial comercial. Int. J. Agric. Nat. Resour. 83-96. Se desarrolló un protocolo de micropropagación para *G. pumila*. Los brotes jóvenes fueron recolectados durante la temporada de crecimiento (octubre a diciembre 2016) de una población silvestre en el área del Volcán Villarrica, in la Región de la Araucanía, Chile. Se utilizaron segmentos nodales para la iniciación *in vitro*, se probaron varios tratamientos de desinfección con diferentes concentraciones de hipoclorito de sodio. Los explantes desinfectados se colocaron en el medio basal WPM100% (WPM100) suplementado con diferentes concentraciones de 2-isopentiladenina (2-iP), con el fin de evaluar el mejor medio de regeneración durante el cultivo *in vitro*. La desinfección se realizó con hipoclorito de sodio al 1% durante 40 minutos, seguida de una segunda desinfección con hipoclorito de sodio al 2% durante 25 minutos y utilizando el medio de cultivo basal MS suplementado con 2mg L^{-1} de 2-iP que proporcionó la mayor eficiencia de las plantas desinfectadas. En la etapa de propagación, las tasas de multiplicación más altas se obtuvieron cuando se agregó 1mg L^{-1} de Zeatina al medio basal WPM100. El enraizamiento y la preaclimatación in vitro fueron mejores cuando los explantes se cultivaron en WPM100 suplementado con 3mg L^{-1} de ácido naftaleno acético. Este protocolo *in vitro* puede ser usado para propagar genotipos de esta especie nativa chilena, además, de ser una herramienta importante para su domesticación y potencial uso.

Palabras clave*:* Enraizamiento *in vitro*, ericaceae, *Gaultheria* sp.*,* micropropagación, reguladores de crecimiento.

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