Elicitation of Phenylpropanoids in Maqui (*Aristotelia chilensis* [Mol.] Stuntz) Plants Micropropagated in Photomixotrophic Temporary Immersion Bioreactors (TIBs).

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Abstract

A biotechnological system for the production of plants biomass and phenylpropanoids of maqui was developed in photomixotrophic TIBs. The *in vitro* maqui multiplication was evaluated using combinations of TDZ and BAP in TIBs 1L capacity. Treatment of MS basal supplemented with TDZ 1 mg/l shows the best results for the variables fresh weight and multiplication rate. Photomixotrophic conditions were standardized in media with 3%, 2%, 1%, 0% sucrose at a light intensity of $100 \mu\text{M m}^{-2}\text{s}^{-1}$. The treatments reduced in sucrose (1% and 2%) and air supplemented with 0.4 MPa CO₂ do not differ significantly in biomass production (fresh weight per cluster of plants) compared to the control treatment with sucrose 3% and standard air. Treatment with ABA (1m/l) induced the production and accumulation of phenylpropanoids metabolites in maqui cultures bioreactors. Phenylpropanoids in *in vitro* biomass of maqui and culture medium from TIBs were determined in parallel with control samples from wild plants and mature fruits. After the third day of analysis, not significant differences in polyphenols and anthocyanin contents were verified between the treatments of maqui in TIBs + ABA and controls. The non-significant differences in the contents of polyphenols and anthocyanin were maintained until the 15 days of analysis. The antioxidant capacity comparing samples of maqui in bioreactors and wild plants showed no significant differences by the ORAC test from day 5 to day 15 of the study.

Key Message

For the first time maqui micropropagation has been established in TIBs followed by elicitation of phenylpropanoids metabolites. Contents of metabolites from maqui in TIBs do not differ from those of wild fruits, while antioxidant capacity of phenylpropanoids was preserved during the study.

1. Introduction

Temporary Immersion Bioreactors (TIBs) technology was introduced some years ago for mass propagation in different plant species (Ziv 2005; Paek et al. 2005). This approach displays some distinctiveness advantages where the most important is the yield improvement during the plant micropropagation process to achieve high-quality production combined with a reduced impact on the environment. Furthermore, this technology allows obtaining pathogen-free plant material, and producing genetically similar (clonal) material in controlled *in vitro* systems (Arencibia et al. 2017). TIBs can be also used in advanced techniques for genetic improvement, *i.e.* to assist the selection during plant genetic engineering (Espinosa et al. 2002).

Particularly, TIBs technology is based on the cultivation of plant cells and tissues in closed bottles but with greater contact with filtered (sterile) air that it can be renewed frequently together with the culture medium. Both plant immersion in the culture medium and air changes follow an automated program depending on the species/genotypes growing in the bioreactor (Ziv et al. 2005). Bioreactors have successfully used as an experimental model for genomic and molecular characterization of the process of releasing phenylpropanoid metabolites into the culture medium during plants micropropagation. In this case, the role of these metabolites in growth, multiplication (biomass production), as well in the *ex vitro* adaptation of micropropagated seedlings have been determined in sugarcane, blueberry, raspberry and, dendroenergetic poplars (Arencibia et al 2008;

2018b). Additionally, TIBs have been useful for obtaining phenylpropanoids as bioproducts with elicitor activity of plant defense mechanisms in tomato and sugarcane (Yang et al. 2010; Arencibia et al. 2012).

In nature, the induction and accumulation of secondary metabolites usually could happen when plants face different biotic and/or abiotic stresses. Taking the example of plant phenylpropanoids it has accepted that their evolution could be relate to the plant response to a spectrum of adverse and selective environmental conditions, as well as the plant requirement of compounds with UV-absorbing and antioxidant properties (Landi et al. 2020). Therefore, high concentration of phenolics could give abiotic tolerance and high level of defense to insect and pathogen attack (Karabourniotis et al. 2014). These processes could be simulated *in vitro* with elicitors or signals molecules which play a key function in the adaptability of plants to overcome a spectrum of stress conditions, *i.e.* drought, salinity, insect or pathogen attack, between others (Arencibia et al. 2008, 2012; Ramakrishna and Gokare 2011; Gai et al. 2020).

Maqui (*Aristotelia chilensis* (Mol.) Stuntz) is a native plant of the Elaeocarpaceae family that grows wild in Central and Southern regions of Chile and Argentina, which produces small purple color berries that are eaten fresh or used for juice and jams (Gonzalez et al. 2015; Rodriguez et al. 2016; Romero 2020). It is an extensive fact that the fruits of plants growing wild in grasslands, mountains, and areas of wild vegetation are collected following traditional practices. Fruits and leaves of maqui have been known in the traditional herbal medicine as beverage treatments for sore throats, kidney pain, digestive ailments, reduce fever, and scarring injuries (Céspedes et al., 2017; Quispe et al. 2020).

In the last years, several scientific research have given demonstrations about the antioxidant, anti-atherogenic, cardioprotective activities, as well as adipogenesis and inflammation inhibitory effects, of mature fruits from maqui plants (Céspedes et al. 2017). High polyphenols content has been associated with these medicinal properties, specifically a spectrum of anthocyanin: delphinidin 3-sambubioside-5-glucoside, delphinidin 3,5-diglucoside, delphinidin 3-sambubioside, delphinidin 3-glucoside, cyanidin 3-sambubioside-5-glucoside, cyanidin 3,5-diglucoside, cyanidin 3-sambubioside, and cyanidin 3-glucoside (Zuñiga et al. 2017).

From this background, the present work is based on the hypothesis that the TIBs technology could be suitable as a circular bioprocess for the production of secondary metabolites of interest to the for high added value industries such as nutraceutical, cosmetic, and pharmaceutical that use natural plant metabolites as sources of raw materials. For the first time, this paper reports the establishment of photomixotrophic TIB_S cultures of maqui (*Aristotelia chilensis* [Mol.] Stuntz), which were induced for phenylpropanoids production. In both fresh biomass and culture medium, the antioxidant metabolites were overproduce after elicitation treatments and its biological activity were similar to those of fruits from ex vitro growing plants. The sustainable production of secondary metabolites from maqui mediated by biotechnological tools is discusses as well as the contributions of this bioindustrial model to a circular bioeconomy.

2. Materials And Methods

2.1 Plant materials

Wild maqui plants (*Aristotelia chilensis* [Mol.] Stuntz) were selected from the surroundings of the locality of Vilches (35° 35' 59" South; 71° 11' 6" West), Maule Region, Chile. A total of 30 individuals were random chosen as donors to the *in vitro* cultures. Nodal segments of the younger upper branches which showed an intense green color were collected during the spring season. In all cases, healthy and non-lignified tissues were selected and kept in moist plastic bags for carrying to the laboratory.

2.2 Establishment of *in vitro* maqui stock

Plant tissues were surface disinfected in a 10% commercial solution NaOCl plus 0.1% Tween 20 (15 min). Explants were immersed in 70% ethanol (5 min.) followed by three rinses in sterile distilled water. The excised shoot tips and nodal segments (~ 1 cm) were cultured for 6 weeks in MS medium (Murashige and Skoog, 1962) supplemented with 1 mg/l BAP (6-Benzylaminopurine), 25 gr/L Sucrose, 7 gr/L Agar, pH 5.6. Cultures were maintained at $23 \pm 2^{\circ}\text{C}$, for a 12 h photoperiod under a combination of both natural light and cool-white fluorescent tubes at a light intensity of $60 \mu\text{M m}^{-2}\text{s}^{-1}$. Regenerated maqui plantlets were subcultured for four months by aseptically transferring shoot segments to the above-described medium.

2.3 Biomass production in bioreactors

Two-vessel programmable bioreactors were set up by following established designs (Yabor et al. 2007; Arencibia et al., 2013 a,b; 2017). Nodal segments from 20 plantlets (two weeks old after subculture) were transferred to a sterile transparent glass bottle (1 L capacity). The couple bottle of the TIBs contained 200 ml of MS basal medium plus sucrose 25 g /l, pH 5.6 with growth regulators. Experimental treatments were designed as follows: **I**- TDZ 1 mg/l for 30 days; **II**- BAP 1 mg/l per 30 days; **III**- TDZ 1 mg/l + BAP 1 mg/l for 30 days; **IV**- BAP 1 mg/l for 15 days followed by TDZ 1 mg/l + BAP 1 mg/l for 15 days; **V**- BAP 1 mg/l for 15 days followed by TDZ 1 mg/l for 15 days. The experiments were replicated six times and bioreactors were maintained at $25 \pm 2^{\circ}\text{C}$ under natural sunlight and cool-white fluorescent tubes at a light intensity of $100 \mu\text{M m}^{-2}\text{s}^{-1}$. Immersion frequency was every 6 hours by 4 minutes. The following variables were determined: fresh weight per cluster (mg), multiplication rate, plant length (cm), and number of internodes per plant. To optimize the photomixotrophic conditions combinations of sucrose (0; 10; 20; 30 gr / L) and CO₂ (standard air; air with 0.4 MPa CO₂) were studied in TIBs of 1L.

2.4 Phenylpropanoids induction in bioreactors

To optimize the induction of phenylpropanoid metabolites in maqui cultures from TIBs, elicitation treatments with ABA (0; 0.5; 1; 2 mg/l) were studied according to Arencibia et al., 2018. Induction with ABA was performed on maqui seedlings of 21-days-old micropropagating in TIBs 1L containing 200 ml MS basal salts without sucrose and air enriched with 0.4 MPa CO₂. Bioreactors were maintained at $25 \pm 2^{\circ}\text{C}$ under natural sunlight and cool-white fluorescent tubes at a light intensity of $100 \mu\text{M m}^{-2}\text{s}^{-1}$. Immersion frequency was every 6 hours by 4 minutes. Three bioreactors were studied for each treatment and the experiment was replicated three times. The spectrophotometric analysis at 320 nm (potential browning) of both the culture medium and the *in vitro* maqui biomass extracts was performed daily for a period of seven days.

2.5 Scale-up of phenylpropanoid production

After three weeks of biomass production in TIBs 1L, culture liquid medium was replaced with 200 ml MS basal salts plus 1 mg/l ABA (abscisic acid), TDZ 1 mg/l, sucrose 10 gr/l, enriched air 0.4 MPa CO₂. Control treatment was MS basal salts without both ABA and sucrose. The production of secondary metabolites was studied in five bioreactors with maqui biomass. For the *in vitro* and *ex vitro* biomass analyzes, the study material was 1 g of fresh weight, while in the liquid samples it was 1 ml of TIB_s medium. The bioreactors were maintained for additional 15 days for phenylpropanoid determinations. Each determination was replicated three times, while the measurements were repeated three times.

The production of phenylpropanoid metabolites of maqui (*Aristotelia chilensis*) was escalated in SETIS™ bioreactors with a capacity of 5L. The experiment was carried out following the optimal conditions previously established, summarizing: Nodal segments from 50 plantlets (two weeks old after subculture) were transferred to a sterile transparent glass bottle. The couple bottle of the TIBs contained 500 ml of MS basal salts with TDZ 1 mg/l, sucrose 10 gr/l, enriched air 0.4 MPa CO₂. After 15 days of biomass production, maqui vitroplantlets were induced with ABA 1 mg/L. The experiment was replicated three times and bioreactors were maintained at 25 ± 2 °C under natural sunlight and cool-white fluorescent tubes at a light intensity of 100 μM m⁻²s⁻¹. Immersion frequency was every 6 hours by 4 minutes.

2.6 Phytochemical characterization

Samples were prepared as follows: In the case of TIBs, 1 mL of culture medium was mixed with 1 mL methanol (HPLC grade). For the metabolites determinations in the biomass, 100 mg fresh tissue from the couple bottle was macerated in 1 mL of methanol. Control samples were mature fruits (100 mg) collected in wild conditions, and foliar tissue (100 mg) from the same plants where the fruits were collected. Biomass samples from wild plants were crushed in a mortar with 1 mL of methanol (HPLC grade). Before analysis, all the samples were filtered through a paper Whatman No 1. Total polyphenol concentration was determined with the Folin-Ciocalteu method and was expressed as mg of gallic acid equivalents per g. Anthocyanins were quantified by the differential pH method (Giusti and Wrolstad, 2001; Lee et al. 2008) using a spectrophotometer (Unicam Helios UV-vis). After 15 min at room temperature the absorbance was measured at 510 and 700 nm, and calculated as (A_{510 nm} - A_{700 nm}) pH1.0 - (A_{515 nm} - A_{700 nm}) pH4.5 Total anthocyanins were expressed as mg of cyaniding 3-glucoside equivalents per g. Antioxidant capacity was measured by OxiSelect ORAC Assay Kit (Cell BioLabs, Inc.) according to the kit protocol and detection performed by a Biotek Syn-ergy HT Fluorescence Micro Plate Reader. ORAC activity was expressed as μmol Trolox equivalents per g.

2.6 Statistical analysis

After checking the homogeneity of the variances by Cochran test (Wilrich, 2013) and the normality of the data by Jarque-Bera test (Thadewald and Büning, 2007), the transformation of the data was conducted by logarithmic transformation and root square transformation. Data were processed with ANOVA, which was significant, and with a post-hoc test with multiple comparisons Turkey-HSD test using the PAST v. 3.17 software for Windows.

Results

Biomass production

Maqui biomass production was standardized in 1L capacity TIBs containing 200 ml of culture medium. Different treatments with the growth regulators TDZ and BAP were studied and variables related to the micropropagation of maqui vitroplants were determined. At 30 days of multiplication, the results show significant differences between the treatments studied in the variables fresh weight (FW) and multiplication rate (MR), while no significant differences were observed in the variables plant length (PL) and number of internodes per plant. The treatment of MS basal medium supplemented with TDZ (1 mg/L) shows the best results for the biomass variables (fresh weight and multiplication rate) with significant differences compared to the studied treatments.

Maqui biomass production was optimized under photomixotrophic conditions in parallel with its scaling in 1L capacity bioreactors. The experiments were carried out with basal MS medium with TDZ (1 mg/L) (previously selected) with combinations of sucrose (0; 10; 20; 30 gr / L) and CO₂ (standard air; air plus 0.4 MPa CO₂).

The results show significant differences in the production of maqui biomass between the treatments (Table 2).

Table 1

Optimization in the production of maqui biomass in a micropropagation cycle (30 days) in 1L TIBs. Data are expressed as an average $\pm$ standard error. (*) Significant differences HSD test ($p < 0.05$).

	Fresh weight per cluster (mg)		Multiplication rate	Length (cm)	Internodes (number/plant)
I-TDZ	37,42 ^a ($\pm$ 3,63)	6,38 ^a ($\pm$ 0,42)	5.83 ($\pm$ 0,53)	4,76 ($\pm$ 0,58)	
II-BAP	28,25 ^b ($\pm$ 3,96)	4,30 ^b ($\pm$ 0,55)	4,76 ($\pm$ 0,63)	4,13 ($\pm$ 0,47)	
III-TDZ + BAP	27,40 ^b ($\pm$ 4,01)	4,54 ^b ($\pm$ 0,74)	6,33 ($\pm$ 0,50)	4,85 ($\pm$ 0,30)	
IV- BAP→TDZ + BAP	24,50 ^b ($\pm$ 5,24)	3,92 ^b ($\pm$ 0,58)	6,54 ($\pm$ 0,46)	4,35 ($\pm$ 0,62)	
V- BAP→TDZ	25,83 ^b ($\pm$ 5,37)		3,65 ^b ($\pm$ 0,67)	5,71 ($\pm$ 0,64)	5,14 ($\pm$ 0,76)
Significance	*		*	ns	ns

Table 2

Biomass production in photomixotrophic conditions during 30 days of culture in TIBs with capacities 1 L and 5 L. Data are expressed as an average $\pm$ standard error. (*) Significant differences HSD test ($p < 0.05$).

Sucrose/ CO ₂	S0		S10		S20		S30		Significance
	Air STD	0.4 MPa	Air STD	0.4 MPa	Air STD	0.4 MPa	Air STD	0.4 MPa	
TIB 1L	11,32 ^c ($\pm$ 1,91)	18,28 ^b ($\pm$ 2,92)	20,54 ^b ($\pm$ 3,85)	34,81 ^a ($\pm$ 3,56)	25,79 ^b ($\pm$ 4,81)	37,73 ^a ($\pm$ 5,12)	36,25 ^a ($\pm$ 4,96)	38,02 ^a ($\pm$ 5,34)	*
TIB 5L	28,94 ^c ($\pm$ 5,53)	39,45 ^c ($\pm$ 6,64)	64,32 ^b ($\pm$ 8,63)	93,47 ^a ($\pm$ 9,65)	74,53 ^b ($\pm$ 8,75)	99,56 ^a ($\pm$ 9,04)	103,42 ^a ($\pm$ 10,43)	102,64 ^a ($\pm$ 9,36)	*

The treatments reduced in sucrose (10 gr/L and 20 gr/L) and supplemented with 0.4 MPa CO₂ do not differ significantly in biomass production (fresh weight per cluster of plants) compared to the control treatment with sucrose 30 gr/L and standard air. The basal MS medium control treatments with sucrose 30 gr/L did not show statistical differences when standard air or air enriched with 0.4 MPa CO₂ was supplied. The treatments without sucrose in the medium had the lowest results in biomass production and the plants showed pale yellowish-greenish coloration at 30 days of cultures in TIBs.

In summary, similar behavior is evidenced for biomass production between the treatments studied in both 1L and 5L bioreactors, additionally, it could be proposed culture media reduced in sucrose (10 gr/L; 20 gr/L), and supplemented with 0.4 MPa CO₂ for productive scaling in maqui photomixotrophic conditions.

Figure 1 shows examples of clusters of maqui plants obtained in 1L TIBs under heterotrophic conditions (basal MS + 30 gr/L of sucrose) with the phytohormones BAP (Fig. 1A) and TDZ (Fig. 1B), observing more vigorous plants and biomass in the culture medium supplemented with TDZ. On the other hand, Fig. 1C shows maqui clusters multiplying in 3L TIBs with medium reduced in sucrose 10 gr/L, enriched air 0.4 MPa CO₂ (photomixotrophic condition) and supplemented with TDZ 1 mg/L. In general, it was possible to show the influence of the *in vitro* environment and the type of culture flask on the phenotype of maqui plants, where characters such as diameter of the stems and size of the leaves can vary, without affecting the survival of the plants and coefficient multiplication in clusters. For this reason, it may be proposed for further studies to increase the number of initial explants per volume of medium in the case of 5L SETIS[™].

Phenylpropanoids induction

The effect of different concentrations of ABA was determined on the induction of maqui phenylpropanoids in TIBs (Fig. 2). The results show the highest absorbance values at 320 nm when the plants were treated with 1 mg/l and 2 mg/l of ABA. Treatment with ABA 0.5 mg/l showed intermediate levels of absorbance, while the control without ABA showed basal values as expected. In addition, it is evident that the levels of phenylpropanoids are higher in the culture medium (Fig. 2A) with respect to the plant biomass (Fig. 2B), showing significant increases between the first 24–48 h for the case of phenylpropanoids released to the

medium of culture, with respect to those accumulated in the biomass of maqui vitroplants. Considering that the absorbance values between 1 mg/l and 2 mg/l of ABA were similar for both culture medium and biomass, the treatment with 1 mg/l ABA was selected for further experiments.

From the previous results, the micropropagation treatment (biomass production) with the most heteromixotrophic condition was selected to induce the production of phenylpropanoid metabolites in bioreactors (total capacity of 1L; 200 mL of culture medium). In this sense, the basal MS culture medium supplemented with TDZ (1 mg/L) and sucrose (10 gr/L) was replaced by basal MS basal plus ABA (1 mg/L) and without sucrose as a source of C. The bioreactors were maintained with enriched air 0.4 MPa CO₂, light intensity of 100 $\mu\text{M m}^{-2}\text{s}^{-1}$, and the immersion frequency was every 6 hours by 4 minutes.

As can be seen in Fig. 3A, in the treatment of maqui plants with ABA (1mg/L) an intense and very dark coloration is evidence in the culture medium that could be associated with phenylpropanoids release. Parallel to the 15 days of induction, the plant biomass cultivated in the BIT also acquires a dark brown almost black color with a shade similar to that of maqui fruits, which can be related to phenylpropanoids accumulation in the plant tissues. The change to a dark brown color begins to be evident first in the culture medium and this occurs from the second day of induction with ABA (Fig. 3B). In the case of biomass, a firstly reddish color change begins to be observe in parts of the foliar tissue, a color that progressively covers the entire biomass over the days, turning dark brown almost black after 15 days of cultivation (Fig. 3C, D).

Phytochemical characterization

For 15 days after the cultures induction with ABA 1mg/L, a phytochemical characterization was conducted in parallel to phenylpropanoid production. The presence of polyphenols and anthocyanin as well as the antioxidant activity were determined in both culture medium and maqui biomass produced in TIBs. The following treatments were used as control: a- mature fruits collected in wild conditions; b: foliar tissue biomass of the plants where the fruits were collected; c: culture medium of bioreactors without ABA induction; d: maqui biomass of bioreactors without ABA induction.

The results of the determination of total polyphenols (Table 3) indicated significant differences between the treatments during the first day after the induction of maqui bioreactors with ABA. From the second day and during the 15 days after, no significant differences were shown between the treatments TIBs + ABA and fruits and foliar biomass extracted from the wild. The contents of total polyphenols were increased in all treatments until days 5th and 7th, while these begin to slightly decrease from 10th and 15th days that was the time of experimental analysis. The results indicate that the levels of polyphenols produced in the TIBs + ABA were similar to those of fruits and wild biomass between the third and seventh day of the study. From this period, phenylpropanoid metabolites may

Table 3

Determination of polyphenols content (g GAE/100 g DW) in TIBs cultures of maqui (*Aristotelia chilensis*). Data are expressed as an average $\pm$ standard error. (*) Significant differences HSD test ($p < 0.05$).

	Days after treatment with ABA							
	1	2	3	4	5	7	10	15
Mature field fruits (C)	2,92 _a ($\pm$ 0,86)	2,96 ^a ($\pm$ 0,85)	2,95 ^a ($\pm$ 0,79)	3,03 _a ($\pm$ 0,88)	3,16 ^a ($\pm$ 0,91)	3,19 ^a ($\pm$ 0,94)	2,92 _a ($\pm$ 0,69)	2,90 _a ($\pm$ 0,81)
Biomass wild plants (C)	2,76 _a ($\pm$ 0,79)	2,79 ^a ($\pm$ 0,76)	2,89 ^a ($\pm$ 0,81)	2,95 _a ($\pm$ 0,76)	2,98 ^a ($\pm$ 0,73)	3,04 ^a ($\pm$ 0,96)	2,87 _a ($\pm$ 0,73)	2,83 _a ($\pm$ 0,75)
Medium TIBs + ABA	0,32 _b ($\pm$ 0,08)	0,93 ^b ($\pm$ 0,21)	2,48 ^a ($\pm$ 0,79)	2,72 _a ($\pm$ 0,81)	3,13 ^a ($\pm$ 1,02)	3,29 ^a ($\pm$ 0,82)	3,07 _a ($\pm$ 0,71)	2,86 _a ($\pm$ 0,79)
Biomass TIBs + ABA	0,57 _b ($\pm$ 0,12)	2,31 ^a ($\pm$ 0,59)	2,34 ^a ($\pm$ 0,65)	2,55 _a ($\pm$ 0,72)	2,83 ^a ($\pm$ 0,89)	2,97 ^a ($\pm$ 0,94)	2,75 _a ($\pm$ 0,71)	2,53 _a ($\pm$ 0,68)
Medium TIBs-ABA (C)	ND	ND	0,06 ^c ($\pm$ 0,01)	0,07 _c ($\pm$ 0,02)	0,10 ^c ($\pm$ 0,05)	0,16 ^c ($\pm$ 0,04)	0,17 _c ($\pm$ 0,06)	0,21 _c ($\pm$ 0,07)
Biomass TIBs-ABA (C)	0,19 _c ($\pm$ 0,07)	0,21 ^c ($\pm$ 0,08)	0,22 ^b ($\pm$ 0,07)	0,27 _b ($\pm$ 0,09)	0,32 ^b ($\pm$ 0,11)	0,38 ^b ($\pm$ 0,09)	0,43 _b ($\pm$ 0,18)	0,57 _b ($\pm$ 0,16)
Significance	*	*	*	*	*	*	*	*

begin to degrade both in maqui plants in TIBs + ABA, and in fruits and biomass collected from wild conditions. The control treatments of maqui plants in TIBs without ABA showed significant differences with the lower levels of polyphenols, which could be considered as basal and in correspondence with the metabolic properties of the maqui.

In parallel, the production of anthocyanin-type secondary metabolites was determined in the TIBs treatments and controls (Table 4).

Table 4

Production of anthocyanin (g cy-3-glu/100 g DW) metabolites in TIBs cultures of maqui (*Aristotelia chilensis*). Data are expressed as an average $\pm$ standard error. (*) Significant differences HSD test ($p < 0.05$).

	Days after treatment with ABA							
	1	2	3	4	5	7	10	15
Mature field fruits (C)	1,83 ^a ($\pm$ 0,70)	1,87 ^a ($\pm$ 0,74)	1,89 ^a ($\pm$ 0,71)	1,89 ^a ($\pm$ 0,68)	1,91 ^a ($\pm$ 0,79)	1,94 ^a ($\pm$ 0,81)	1,93 ^a ($\pm$ 084)	1,88 ^a ($\pm$ 0,75)
Biomass wild plants (C)	1,69 ^a ($\pm$ 0,66)	1,71 ^a ($\pm$ 0,52)	1,73 ^a ($\pm$ 0,64)	1,75 ^a ($\pm$ 0,71)	1,84 ^a ($\pm$ 0,73)	1,85 ^a ($\pm$ 0,68)	1,81 ^a ($\pm$ 0,77)	1,72 ^a ($\pm$ 0,60)
Medium TIBs + ABA	0,15 ^b ($\pm$ 0,05)	0,69 ^b ($\pm$ 0,16)	1,39 ^a ($\pm$ 0,51)	1,68 ^a ($\pm$ 0,59)	1,72 ^a ($\pm$ 0,64)	1,78 ^a ($\pm$ 0,74)	1,72 ^a ($\pm$ 0,82)	1,71 ^a ($\pm$ 0,69)
Biomass TIBs + ABA	0,21 ^b ($\pm$ 0,08)	0,82 ^b ($\pm$ 0,28)	1,31 ^a ($\pm$ 0,46)	1,59 ^a ($\pm$ 0,58)	1,67 ^a ($\pm$ 0,62)	1,69 ^a ($\pm$ 0,79)	1,65 ^a ($\pm$ 0,68)	1,63 ^a ($\pm$ 0,73)
Medium TIBs-ABA (C)	ND	ND	0,03 ^c ($\pm$ 0,02)	0,04 ^c ($\pm$ 0,02)	0,05 ^c ($\pm$ 0,01)	0,06 ^c ($\pm$ 0,02)	0,07 ^c ($\pm$ 0,03)	0,07 ^c ($\pm$ 0,02)
Biomass TIBs-ABA (C)	0,08 ^c ($\pm$ 0,02)	0,09 ^c ($\pm$ 0,01)	0,11 ^b ($\pm$ 0,04)	0,11 ^b ($\pm$ 0,03)	0,22 ^b ($\pm$ 0,05)	0,28 ^b ($\pm$ 0,09)	0,33 ^b ($\pm$ 0,1)	0,37 ^b ($\pm$ 0,09)
Significance	*	*	*	*	*	*	*	*

In general, the contents of anthocyanin were lower than the total phenols in all treatments during the experiment (15 days). The levels of anthocyanin were significantly lower (first evaluation-1 day) in the two treatments of maqui in TIBs compared to the controls of fruits and wild biomass. The results also show that from the third day of induction with ABA, the levels of anthocyanin do not differ significantly between the TIBs + ABA treatments and the wild controls. For all the treatments studied, the highest levels of anthocyanin occur 7 days after induction, evidencing a slight decrease from the 10th day of induction. The control treatments maqui TIBs without ABA showed significant differences with the lower (basal) levels of anthocyanins, in correspondence with previous polyphenols determination.

To verify the biological propriety useful for the food industry of the phenylpropanoid metabolites produced in TIBs, the antioxidant activity was determined by the ORAC assay in the previously described treatments and controls (Table 5). The results showed significant differences between the treatments with the highest values of antioxidant activity from the first day of analysis in the samples of fruits and biomass of wild plants. In the

bioreactor treatments with maqui plus ABA, the antioxidant activity values increase, reaching non-significant differences on the 4th day when compared to the wild maqui controls. From the 5th day to the 15th day of analysis, both maqui treatments (culture medium and biomass) have high values of antioxidant activity, which do not differ significantly from the antioxidant activity of the controls of fruits and leaves collected from wild plants. As expected, the control treatments of maqui bioreactors without induction with ABA showed basal levels of antioxidant activity in the case of biomass, being even lower in the culture medium that could only be determined after the 4th day of analysis.

Table 5

Quantification of antioxidant activity by ORAC assay ($\mu\text{mol Trolox/g DW}$) in extracts of maqui (*Aristotelia chilensis*) cultured in TIBs. Data are expressed as an average $\pm$ standard error. (*) Significant differences HSD test ($p < 0.05$).

	Days after treatment with ABA							
	1	2	3	4	5	7	10	15
Mature field fruits (C)	10,93 ^a ($\pm$ 2,96)	10,97 ^a ($\pm$ 2,25)	11,04 ^a ($\pm$ 2,62)	11,35 ^a ($\pm$ 2,43)	11,39 ^a ($\pm$ 2,58)	11,49 ^a ($\pm$ 1,98)	11,45 ^a ($\pm$ 2,56)	11,38 ^a ($\pm$ 2,79)
Biomass wild plants (C)	9,69 ^a ($\pm$ 2,08)	9,71 ^a ($\pm$ 2,63)	10,13 ^a ($\pm$ 2,76)	10,19 ^a ($\pm$ 2,18)	10,21 ^a ($\pm$ 2,33)	10,05 ^a ($\pm$ 2,05)	10,03 ^a ($\pm$ 2,31)	9,99 ^a ($\pm$ 1,98)
Medium TIBs + ABA	2,45 ^b ($\pm$ 0,88)	4,97 ^b ($\pm$ 1,58)	6,85 ^b ($\pm$ 1,09)	7,99 ^{ab} ($\pm$ 2,41)	9,56 ^a ($\pm$ 2,09)	10,59 ^a ($\pm$ 2,37)	10,99 ^a ($\pm$ 2,83)	10,42 ^a ($\pm$ 1,75)
Biomass TIBs + ABA	1,91 ^b ($\pm$ 0,66)	3,72 ^b ($\pm$ 1,44)	5,61 ^b ($\pm$ 1,31)	6,79 ^b ($\pm$ 1,87)	9,03 ^a ($\pm$ 1,66)	9,45 ^a ($\pm$ 1,39)	9,55 ^a ($\pm$ 2,01)	9,26 ^a ($\pm$ 1,84)
Medium TIBs-ABA (C)	ND	ND	ND	0,05 ^d ($\pm$ 0,01)	0,16 ^c ($\pm$ 0,03)	0,29 ^c ($\pm$ 0,07)	0,54 ^c ($\pm$ 0,14)	0,51 ^c ($\pm$ 0,16)
Biomass TIBs-ABA (C)	1,72 ^b ($\pm$ 0,52)	1,79 ^b ($\pm$ 0,61)	1,82 ^c ($\pm$ 0,54)	1,86 ^c ($\pm$ 0,68)	1,91 ^b ($\pm$ 0,59)	2,04 ^b ($\pm$ 0,58)	2,18 ^b ($\pm$ 0,63)	2,14 ^b ($\pm$ 0,76)
Significance	*	*	*	*	*	*	*	*

Scaling-up production.

The bioreactor workstation was designed and built to be flexible and adaptable to different models of TIBs with variable capacity. The station has been standardized under a combination of natural light and artificial light, achieving a luminous intensity of $100 \mu\text{M m}^{-2}\text{s}^{-1}$ that allows the establishment of photomyxotrophic *in*

vitro cultures. In parallel and according to our previous results, the air was enriched with 0.4 MPA CO₂ while sucrose was reduced to 10 gr/L in the culture medium. Considering that both biomass production and phenylpropanoid induction was conducted in TIBs 1L total volume (100 ml culture medium) and in round commercial bottles (Fig. 3A), the first scaling step was designed in TIBs model SETIS with a total volume of 5L of rectangular shape (Fig. 4), allowing to optimize the productive spaces of the workstation.

The results of the scaling in the production of maqui phenylpropanoid metabolites are shown in Fig. 4. According to the previous results in 1L bioreactors, no evident change in color was observed in the culture medium in the control of maqui plants without induction with ABA (Fig. 4A). In parallel, a significant darkening was evidenced in the culture medium corresponding to the bioreactors with maqui plants induced with 1 mg/L of ABA (Fig. 4B; C).

The scaling up to 5L bioreactors allowed us to determine the reproducibility of the production of phenylpropanoids from maqui. However, some observations suggest that this process can be improved in its production efficiency. For example, it was observed that the color change to red-brown occurs first in the culture medium up to 10 days of study, while in the *in vitro* biomass this change is slower and begins to be evident after 15 days of induction. with ABA.

This in contrast to the 1L bioreactor where at 15 days the biomass was completely dark brown to black in color characteristic of maqui metabolites. This observation could indicate the influence of the *in vitro* environment (space and distribution), as well as the potential to increase the initial inoculum/ culture medium ratio as a way to increase yields, both in the release to the culture medium, as in the accumulation in plant tissues of phenylpropanoid metabolites.

Discussion

This work has the novelty of standardizing the production of phenylpropanoid secondary metabolites from whole maqui plants grown in TIBs of 1L. Results originate an optimized methodology that was scale to bioreactors of 5L capacities. It is the first time that micropropagation of maqui is carry out in TIBs, in this case, to obtain plant biomass for the production of natural secondary metabolites for potential commercial use in the food industry. On the other hand, it is note that the secondary metabolites produced in TIBs originate from the biosynthesis of maqui vitroplants with an active potential photosynthetic. Therefore, we can infer that the optimization of photomixotropic plant bioreactors should improve the photosynthetic processes responsible for the natural biosynthesis of biomolecules of nutritional value (Arencibia et al. 2018a). The results obtained could be considered biologically efficient and should be an alternative to avoid the use of more complex production systems, *i.e.* those mediated by recombinant microorganisms and/or plants or other metabolic engineering strategies including synthetic organic chemistry (Carqueijeiro et al. 2020; Cho et al. 2020; Yang et al. 2020).

Traditionally the systems for the *in vitro* production of secondary metabolites have been based on the use of plant cell suspensions and callus, for that are limited to satisfy the commercial requirements of a narrow range of metabolites (Eibl and Eibl, 2008). Secondary metabolite production by cell cultures is an alternative to chemical synthesis however; it may be advantageous only when the metabolites are biosynthesized in specific

plant tissues. Examples have been reported in carrot, mung beans, safflower, sunflower, saffron, wormwood and yew (Caretto et al. 2011; Malik et al. 2011; Yue et al. 2016; Almagro et al. 2017; Han et al. 2020; Moradi et al. 2020). With the aim of increasing the perspectives and widening the horizons in this field, the systems for the production of secondary metabolites using *in vitro* cell cultures are under particular consideration and critically studied (Sajc et al. 2000, Zhong, 2001; Ruffoni et al. 2010). Our proposal to use photosynthetic active plants in bioreactors could become a viable alternative to the limitations of the use of plant cell cultures and calluses in the production of secondary metabolites for commercial use.

The induction of metabolites took place by adding ABA to the concentration of 1 ml/l after three weeks of maqui biomass growth in photomixotrophic conditions. The abscisic acid (ABA) is considered an inhibitor of plant growth and is usually used as a retarder in the growth of plant tissue cultures (Rai et al. 2011). However, several authors have pointed out that while the exogenous ABA alone suppresses the regeneration of the shoots, but if it is added to a plant culture system in combination with other growth regulators, it acts as a promoter in regeneration and growth (Von Arnold et al. 2002; Rai et al. 2011).

Recently, the transcriptomic and metabolomic analysis revealed that exogenous ABA induced the expression of genes involved in flavonoid biosynthesis and lipid metabolism (Gai et al. 2020). Progress elucidating the ABA biosynthesis and the roles of ABA on plant-water relations and on primary and secondary metabolisms were reviewed during the sucrose metabolism and drought stress (Thalmann et al. 2016; Cardoso et al. 2020). Several key genes of the flavonoid biosynthetic pathway were confirmed to be up-regulated both in fruits and leaves in relation to rises in the ABA content throughout simulate drought in grape (Villalobos et al. 2016; Murcia et al. 2017). In strawberry (*Fragaria ananassa* cv. Camarosa) the abiotic stresses (drought and salt) resulted in an increased content of phenolics, anthocyanins, and l-ascorbic acid, in parallel to increased antioxidant activity (Perin et al. 2019).

In this research, the production of secondary metabolites was induced by ABA during the final stage of maqui micropropagation in TIBs. Bioreactors are a technological tool where plants multiply and increase their biomass in contact with air (in this case enriched with CO₂) and immersion in culture medium occurs temporarily every fixed time intervals of 6 hours. This increase in biomass at fixed immersion intervals could cause slight drought stress over time, which in interaction with the exogenous supply of ABA could explain the production and release of phenylpropanoid metabolites by the biomass of maqui plants. This explanation paves the way for further improvements in the production levels of phenylpropanoids by lengthening the immersion periods, therefore increasing the water stress pressure in the plants multiplying in the bioreactors.

Another important point has been the use of a combination of natural and artificial light, achieving increases in light intensities in *in vitro* conditions to implement photomixotrophic cultures. This parameter was initially optimized for the case of bioreactors focus on micropropagation of sugarcane, raspberries, dendroenergetic poplars, and blueberries (Arencibia et al. 2012; 2013a,b; 2017) and, in the course of the experiments was consequent to support the production of secondary metabolites in blueberries (Arencibia et al. 2018a). In a recent example, influences of combinations of blue, red, and white light were studied in the medicinal plant *Dysphania ambrosioides* L. (De Carvalho et al. 2020). The role of light quality in biotechnological processes related to *in vitro* plant culture systems has been reviewed indicating their influence on plant metabolism, growth and development (Arencibia et al. 2018b; Batista et al. 2018). Otherwise, response to monochromatic

light environments reveals a plasticity of photosynthetic processes regarding the accumulation of secondary metabolites in plants (Landi et al. 2020).

Referring to applications the spectrum of light, in the last years some research points to the use of UV-B as an elicitor to rise the production of secondary metabolites under controlled conditions (Dou et al. 2019; Zhang and Jiang, 2019). For example, a study on the effect of preharvest UV-C treatment on the physiology of strawberry fruits demonstrated an attenuated fruit postharvest senescence and augmented phytochemicals profile (Xu et al. 2019). In the case of biomass, a report on basil concluded that the application of UV-B enhance the post-harvest nutraceutical quality of leaves and their antioxidant potential because of an increase in the content of polyphenols (Nascimento et al. 2020). These antecedents pave the way for further improvements in the efficiencies in the production of phenylpropanoids obtained in this work. Bioreactor stations have the flexibility that would allow experiments with different light spectra, including ultraviolet light, applied to photosynthetic active plant biomass under controlled conditions.

In this work, it is demonstrated that the levels of phenylpropanoid metabolites obtained in ABA-induced maqui TIBs are comparable to those of fruits and leaves of maqui plants harvested from natural conditions. As a relevant point, the antioxidant activity from the 5th day and until the end of the experiment at 15 days was also similar between the fruits and maqui biomass under natural conditions and, those obtained from maqui biomass in bioreactors supplemented with ABA. It is explained that this study is based on the standard micropropagation cycle of maqui in TIBs, which in this final stage includes both the period of increase in plant yield and the induction period of the rooting stage. These results are promising and pave the way for further research on formulation variants that consider bioavailability studies of phenylpropanoids with an emphasis on the nutraceutical and functional food industry.

Different approaches are being studied to standardize techniques that allow optimizing the biological activity and bioaccessibility of phenylpropanoids (Hemaiswarya and Doble, 2010; Baenas et al. 2016; Swieca, 2016). A microencapsulated anthocyanin (cyanidin-3-*O*-galactoside from Saskatoon berries) by using a deep eutectic solvent (DES) demonstrated significantly improved stability parameters in response to changes in light, oxygen, moisture content and pH (Zeng et al. 2018). Recently an improved of antioxidative properties of breads was reported after supplementation with microencapsulated powder of Saskatoon berry covered with maltodextrin and inulin (Lachowicz et al. 2021). In the case of maqui, a leaf extracts with high phenolic compound levels were microcapsulated in water-in-oil emulsion (W/O) using a phase of the aqueous, gum arabic, maltodextrin/inulin and a liquid vaseline phase (Vidal et al. 2013; Romero et al. 2020). In other order, encapsulation using *Saccharomyces cerevisiae* is an approach to preserve the bioactivity and increase the stability of functional bioactive compounds (Karaman, 2020). In perspective, the results of the production of phenylpropanoid metabolites from *in vitro* cultures of maqui in bioreactors should allow the technology developed in this work to be more efficiently integrate with technological systems for purification and formulation of bioactive molecules of maqui for commercial use.

In summary, this work attempts to establish a bioeconomic model starting from the design of the biosynthesis process of bioactive compounds of plants with high value (Aguilar et al. 2013; Lokko et al. 2018; Awasthi et al. 2019). The contributions to this model will be explained below. First, the use of relatively few meristematic maqui explants extracted from wild conditions should have a greater a positive impact on the conservation of

wild biodiversity (Uzohab and Babalola, 2018), compared to the current processes of extensive overexploitation of fruits that frequently accelerate the destruction of the wild plantations. Second and in the same line, the scaling of biomolecules production systems in bioreactors will diminish the importance and justification for the implementation of costly and long-term genetic improvement programs for this native species. It is indicate that the potential selection of clones or improved varieties would impose increased competition for two limiting resources in modern agriculture: agricultural land and stable availability of water. Third, the standardization of photomixotrophic cultures in bioreactors increases the efficiency of photosynthesis and reduces electricity consumption. This point still has the potential to be optimized until reaching autotrophic *in vitro* cultures that, coupled with the use of solar panels, would allow obtaining green electricity that can be used for the operation of bioindustrial components (Karan et al. 2019; Bathia et al. 2021). Fourth, the bioreactors work with enriched air + CO₂, producing maqui phenylpropanoids that accumulate in the biomass *in vitro* and are released in parallel to the culture medium, therefore they constitute two sources of raw materials for further purification and formulation processes without residuals for this stage (Barragán-Ocaña et al. 2020).

Declarations

Compliance with ethical standards

Conflict of interest.

The authors declare that they had no conflict of interest. All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Author contributions.

GET performed the establishment of maqui cultures in TIBs and the subsequent elicitation of phenylpropanoids. MR conducted the study of photomixotropic conditions. DG and EV performed the scaled assays and phytochemical analyzes. DA performed phytochemical and data analysis. AG made the introductions of maqui to *in vitro* culture, material preparations and solutions. LA and ADA carried out the general design of the project, analysis and interpretation of data, as well as the writing and revision of the manuscript.

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Figures

Figure 1

Biomass production of maqui (*Aristotelia chilensis*) in TIBs. A; B: Micropropagation (biomass production) of maqui in TIBs with culture mediums supplemented with BAP or TDZ, respectively. C: Scaling up in the production of maqui biomass in commercial temporary immersion bioreactors (SETIS™) of 5L total volume.

Figure 2

Spectrophotometric analysis at 320 nm (potential browning) of phenylpropanoids from maqui (*Aristotelia chilensis*) plants micropropagated in TIBs. A: Phenylpropanoids released to culture medium after elicitation with ABA. B: Phenylpropanoids accumulated in plants biomass after elicitation with ABA.

Figure 3

Production of phenylpropanoids from maqui (*Aristotelia chilensis*) biomass in photomixotrophic TIBs 1L volume. A: Treatment of maqui plantlets with ABA induces both phenylpropanoids releasing into the culture medium and, phenylpropanoids accumulation in plant biomass. B: Phenylpropanoids released to culture medium within two days after the induction with ABA. C; D: Close-up of the foliar tissue biomass showing first a reddish-purple color that progressively changes to black-brown with the days in cultivation.

Figure 4

Scaling up the phenylpropanoid production from maqui (*Aristotelia chilensis*) in vitro cultures in photomixotrophic SETIS™ Temporary Immersion Bioreactors (TIBs). A: Maqui in vitro culture without ABA induction (Control treatment). B-C: Replicas of TIBs evidencing the brown dark color of phenylpropanoids release to the culture medium in maqui plantlets after 7 days of induction with ABA (1mg/L).