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MICROPROPAGATION OF *ZUCCAGNIA PUNCTATA*, AN ARGENTINE MEDICINAL PLANT, BY MEANS OF "IN VITRO" TECHNOLOGY TO PROMOVE ITS CONSERVATION AND THE SUSTAINABLE PRODUCTION OF BIOACTIVE COMPOUNDS

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Abstract. Álvarez, M. A.; C. Pedraza Kobak, R. J. Enrico, F. C. Danert, I. C. Zampini & M. I. Isla. 2024. Micropropagation of *Zuccagnia punctata*, an argentine medicinal plant, by means of "in vitro" technology to promove its conservation and the sustainable production of bioactive compounds. *Darwiniana*, nueva serie 12(1): 135-148.

Zuccagnia punctata Cav. ("jarilla poposa") is a monotypic argentine native medicinal plant used by indigenous communities for the treatment of several pathologies. The pharmacological properties have been scientifically validated and its potential use in phytocosmetic and pharmaceutic industry as anti-aged, hypoglycemic, anti-inflammatory, antitumoral, antioxidant, antibacterial and antimicotic has been previously demonstrated. However, the species has a low propagation rate in natural conditions and has been included in the preliminary red list of endangered plants in category 3. Therefore, it is necessary to develop methodologies leading to its conservation and propagation to achieve its sustainable use. The objective of this work is to perform a micropropagation protocol for *Z. punctata* species for the sustainable use and conservation of this species. Furthermore, the study evaluated also the impact of "in vitro" propagation on the content of bioactive compounds of ethanolic extracts obtained from *Z. punctata* plants obtained "in vitro" with collected wild plants. Seeds have been used as mother material. The seed germination to obtain explants were performed on Murashige and Skoog medium (MS) without plant growth regulators (PGRs) containing sucrose, agar, and ascorbic acid. The same medium was employed for the multiplication of shoots. "In vitro" rooting was achieved in liquid and semisolid medium, and a higher yield was obtained in the latter. The plants were transferred into commercial substrate and acclimatized in a greenhouse. The micropropagated plants (MP) had a high content of total phenolic compounds similar to those obtained from wild adult plants (WAP) collected in the Monte Desert. The flavonoid content of MP was higher than that of WAP. The main chalcones identified in WAP, 2′,4′ dihydroxychalcone (DHC) and 2′,4′-dihydroxy-3′-methoxychalcone (DHMC) were also present in the MP with a rate 1/1, and 1/3 in WAP. The extracts obtained from the MP showed antioxidant, anti-inflammatory and antimicrobial activity, similar to WAP. The "in vitro" propagation and acclimatization of *Z. punctata* constitutes a fundamental tool to achieve large-scale multiplication of this species in the future and the sustainable production of bioactive compounds with commercial applications. Reimplantation in the natural habitat could prevent the loss of this medicinal genetic resource.

Keywords. Argentina Medicinal plant; chemical composition; jarilla poposa; micropropagation; sustainable production.

Resumen. Álvarez, M. A.; C. Pedraza Kobak, R. J. Enrico, F. C. Danert, I. C. Zampini & M. I. Isla. 2024. Micropropagación de *Zuccagnia punctata*, planta medicinal argentina mediante tecnología "in vitro" para impulsar su conservación y producción sostenible de compuestos bioactivos. *Darwiniana*, nueva serie 12(1): 135-148.

Zuccagnia punctata Cav. ("jarilla poposa") es una planta medicinal nativa argentina monotípica utilizada por las comunidades indígenas para el tratamiento de diversas patologías. Las propiedades

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farmacológicas fueron validadas científicamente y previamente se demostró su potencial uso en la industria fitocosmética y farmacéutica como antienvejecimiento, hipoglucemiante, antiinflamatorio, antitumoral, antioxidante, antibacteriano y antimicótico. Sin embargo, la especie tiene una baja tasa de propagación en condiciones naturales y ha sido incluida en la lista roja preliminar de plantas en peligro de extinción en la categoría 3. Por ello, se requiere desarrollar metodologías que permitan su conservación y propagación para lograr un uso sostenible. El objetivo de este trabajo es realizar un protocolo de micropropagación de la especie *Z. punctata* y evaluar el efecto de la propagación "in vitro" sobre el contenido de compuestos bioactivos en extractos etanólicos de plantas micropropagadas y plantas adultas salvajes colectadas a campo. Para ello se utilizaron semillas como material madre. La germinación de las semillas para la obtención de explantos se realizó en medio Murashige y Skoog (MS) sin reguladores de crecimiento conteniendo sacarosa, agar y ácido ascórbico. Se utilizó el mismo medio para la multiplicación de los brotes. El enraizamiento "in vitro" se logró en medio líquido y semisólido, logrando mayor rendimiento en este último. Las plantas se transfirieron en sustrato comercial y se aclimataron en un invernadero. Las plantas micropropagadas (PM) tuvieron un alto contenido de compuestos fenólicos totales similares a los obtenidos de plantas adultas silvestres (PAS) recolectadas en el desierto de Monte. El contenido de flavonoides de las PM fue mayor que el de las PAS y las principales chalconas identificadas en PAS, 2′,4′ dihidroxichalcona (DHC) y 2′,4′-dihidroxi-3′-metoxichalcona (DHMC) también estuvieron presentes en las plantas micropropagadas en una proporción de 1/1, mientras que en PAS fue de 1/3. Los extractos obtenidos de las PM presentaron actividad antioxidante, antiinflamatoria, y antimicrobiana similares a las PAS. La propagación "in vitro" y aclimatación de *Z. punctata* constituye una herramienta fundamental para lograr en el futuro la multiplicación a gran escala de esta especie y la producción sostenible de compuestos bioactivos con aplicaciones comerciales. La reimplantación en el hábitat natural podría evitar la pérdida de este recurso genético medicinal.

Palabras clave. Planta medicinal argentina; Composición química; jarilla poposa; micropropagación; producción sustentable.

INTRODUCTION

Zuccagnia punctata Cav. (Fabaceae, Caesalpiniaceae) is an Argentine medicinal glutinous and aromatic shrub, popularly called "jarilla poposa", "jarilla pispito", "pus pus", "lata", and "jarilla macho" (Cabrera, 1976; Ratera & Ratera, 1980; Toursarkissian, 1980; Zuloaga & Morrone, 1999; Ulibarri, 2005; Ladio & Lozada, 2009) (Fig. 1A). Two chemotypes have recently been described, one with reddish-brown fruits and other with yellow fruits (Álvarez et al., 2023). Both are capsuliform, indehiscent and uniseminated (Burkart et al., 1999; Zuloaga & Morrone, 1999; Ulibarri, 2005) (Figs. 1B and C).

This shrub has numerous uses in folk medicine; in fact, the infusion and decoction of aerial parts of jarilla poposa have been widely used in Argentina as foot antiseptic and rubefacient, as well as against bacterial and fungal infections, asthma, arthritis, rheumatism, inflammations, and tumors (Isla et al., 2021). Several pharmacological properties, namely, antibiotic, antiulcerogenic, antifungal, anti-inflammatory, antihypertensive, hypolipemiant, hypoglicemiant, antioxidant, inhibitor of chemotherapeutic resistance factors such as efflux pump and anti-age enzyme inhibitor have been demonstrated (Chieli et al., 2012; Isla et al., 2016; 2021a, 2021b; Moreno et al., 2018; Orqueda et al., 2021; Valoy et al., 2023a, 2023b). Oral administration of jarilla poposa extract and isolated compounds, i.e. chalcones and flavonoids, obtained from them was not toxic in models with rabbits and mice (Isla et al., 2021a, 2021b; Valoy et al., 2023a, 2023b). No genotoxic effect of jarilla poposa extracts was demonstrated (Zampini et al., 2008). *Zuccagnia punctata* could be considered an herbal medicine of traditional use (Isla et al., 2021). The potential of the jarilla poposa extracts in food products (Correa Uriburu et al., 2023) has recently been demonstrated. In other words, this species from arid regions of Argentina has a great potential for its industrialization in food, cosmetics and pharmacy but to date, it is considered a neglected or underutilized species; its inclusion in cropping systems may improve production sustainability, as it may improve biodiversity and climate adaptability, while also decreasing the environmental footprint.

Our purpose is to move such species from a "neglected and underutilized" state to a cultivated state, while expanding the spectrum of species used for the care of human health, particularly in arid, semi-arid and marginal areas, in the context of climate change. Therefore, it is necessary to focus not only on the chemical-pharmacological characterization of species for the valorization of bioactive compounds, but also on propagation

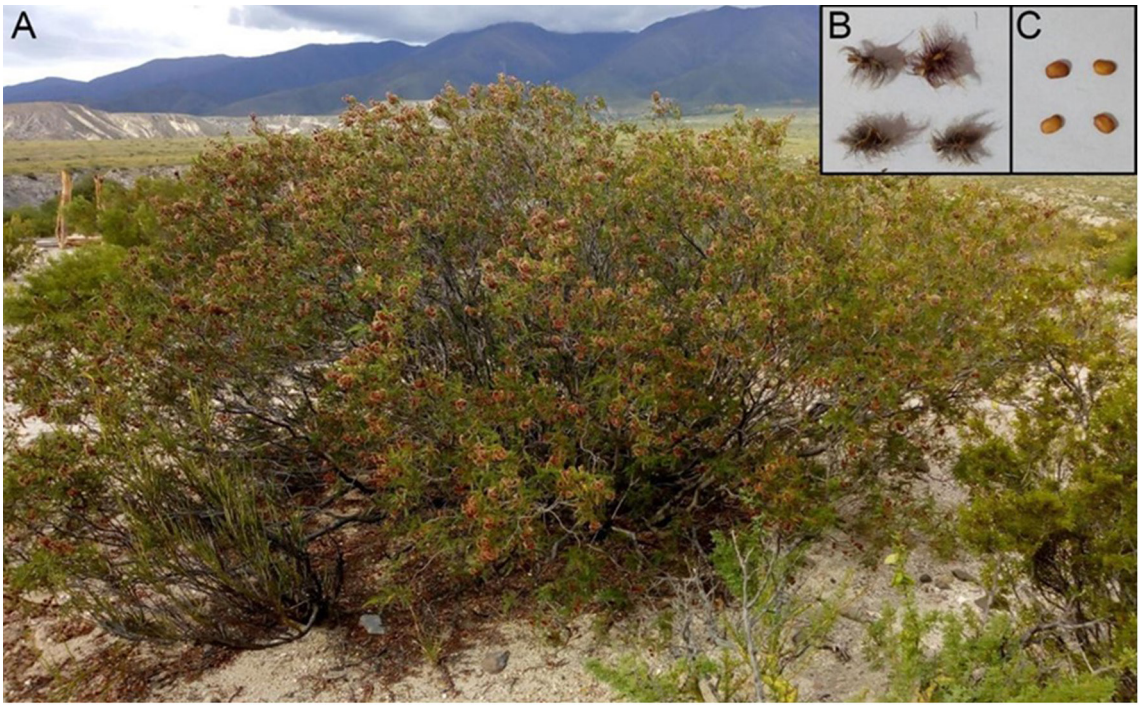

Fig. 1. A. *Zuccagnia punctata* in Amaicha del Valle; **B.** Mature fruits; **C.** Seeds.

methods, designed to counteract destructive collection procedures. In addition to the fact that urbanization and desertification are advancing in the environments where *Z. punctata* grows and fewer populations of the species are available, poor germination of *Z. punctata* seeds is observed in the field (unpublished data). Also, the absence of seeds of this species under the canopy of *Z. punctata* plants or other shrubs that grow in the Monte desert is also noticeable as it was demonstrated (Varela et al., 2021). It is due to all these hardships that *Z. punctata* has been included in the Argentina preliminary red list of endangered plants in category 3 (Norma RE-84-2010-SADS).

Limited knowledge exists with regards to the micropropagation of native xerophytic shrub species from arid and semiarid regions of Argentina. In this context, techniques based on plant tissue culture have appeared as an alternative for the multiplication, conservation, and "in vitro" production of high-value plant species' secondary metabolites worldwide. The present work deals with a micropropagation protocol for this species developed for the first time. The findings emerging from here could be utilized for "in vitro" and "ex vitro" conservation, multiplication, and sustainable production of bioactive compounds to obtain phytocosmetic, phytopharmaceutic and other products from jarilla poposa. So, the study evaluated also the impact of "in vitro" micropropagation on the content of bioactive compounds of ethanolic extracts obtained from *Z. punctata* plants obtained "in vitro" with wild plants.

MATERIALS AND METHODS

Plant material

Mature reddish-brown fruits of *Z. punctata* were collected in Amaicha del Valle, Departamento Tafí del Valle, Tucumán, Argentina at 2556 m a.s.l. (26º 35' 30.24'' S; 65º 52' 48.42'' W) (Fig. 1B). These fruits were stored in dry paper bags at room temperature (25 °C) until use. A classification of seeds based on size (between 0.1-0.3 cm; 0.3-0.6 cm; 0.6-0.8 cm) was performed obtaining three groups, GI, GII and GIII, respectively (Fig. 2).

The seeds were disinfected by immersion in 70° ethanol for one min and then in 15% sodium hypochlorite solution for 20 minutes with continuous shaking in laminar air flow cabinet. Then, the seeds were immersed in sterile distilled water (20 mL in 100 mL Erlenmeyer flasks) on a rotary shaker (100 rpm) in the dark for five minutes and washed three times to remove traces of sodium hypochlorite. The aseptic seeds were cultured "in vitro" conditions to obtain explants. The germination power (GP) of seeds was calculated.

Basal Culture medium

Half-strength Murashige and Skoog medium (Murashige & Skoog, 1962) containing 30 $g.L^{-1}$ sucrose and 100 $mg.L^{-1}$ ascorbic acid as antioxidant was used as basal culture medium (BM). The pH was adjusted to 5.80 ± 0.02. The culture medium (4 mL) was put in glass tubes (18 x 90 mm) and was sterilized by autoclaving at 120 °C and 1 $kg.cm^{-2}$ pressure for 20 minutes.

Establishment of seedlings from "in vitro" seed germination

Aseptic seeds (GIII) were cultured "in vitro" on BM with or without plant growth regulators (PGRs), 0.5 $mg.L^{-1}$ 6-Benzylaminopurine (BAP) and 0.1 $mg.L^{-1}$ Indole-3-butyric acid (IBA) and added with 0.75 $g.L^{-1}$ of agar for 60 days at 26 ± 2 °C under a 16 h photoperiod and 112 µmol m^{-2} s^{-1} provided by cool white fluorescent light (Phillips). The germination percentage, and bud number of seedling was determined. Microcuttings with one axillary bud obtained from "in vitro" seedling was used as explants for micropropagation. For the establishment of seedlings from "in vitro" seed germination, the experiments were carried out with 200 seeds in each treatment x 3 repetitions.

"In vitro" induction and multiplication of shoots

The explants obtained were cultivated in BM without and with different combinations of PGRs, BAP (between 0 and 4 $mg.L^{-1}$) and IBA (0 and 0.5 $mg.L^{-1}$) and added with 0.75 $g.L^{-1}$ of agar (Table 1). The cultures were kept for 30 days under the same conditions of establishment stage. Then, the regenerated multiple shoots (at least 3 cm in length) were sub-culturing on the same fresh medium in similar conditions.

For shoot multiplication experiments, two trials with 50 replicates in each treatment were performed.

"In vitro" rooting of the shoots and acclimatization

After 30 days of culture in multiplication medium, *Z. punctata* individual shoots from 1 to 3 cm were separated and placed in rooting culture medium (liquid BM with 10 $mg.L^{-1}$ IBA), and kept for 15 days in the culture room; then, one part was transferred to liquid BM containing IBA (0.5 $mg.L^{-1}$) and the other part to semi-solid BM with the same composition of liquid medium with agar (0.50 $g.L^{-1}$) for 56 days at the same conditions that previous culture stages. The rooting experiments were carried out with 20 repetitions in each medium x 2 repetitions.

Acclimatization of the plants was done in a growth room equipped with full spectrum LED lights; the plants placed in pots with a commercial substrate (Growmix) composed by fine fiber of Moss peat, fine crust compost, perlite, pH corrector and fertilizers, and kept covered with transparent plastic for 20 days. The cover was gradually removed until the plant was completely exposed.

Foliar anatomical studies of shoots obtained by "in vitro" culture

Experiments were conducted to study the anatomical changes in leaves of shoots developed "in vitro" during the multiplication stage. The diaphanization were carried out according to the Dizeo technique of Strittmatter (1973).

Leaflets of *Z. punctata* were embedded in 3% agarose (type II) before cutting for anatomical examination. Cross-sections (25 µm) were obtained by using a microtome and stained following conventional histological methods (Mercado & Ponessa 2021) and were observed under the light microscope.

Fig. 2. Seeds of *Zuccagnia punctata* GI, GII and GIII of different sizes, shapes and colors.

Table 1. Different combinations of plant growth regulators IBA (Indole-3-butyric acid) and BAP (6-Benzylaminopurine).

Culture Media	IBA ($mg.L^{-1}$)	BAP ($mg.L^{-1}$)
1	0	0
2	0	1
3	0	2
4	0	4
5	0.1	0
6	0.1	1
7	0.1	2
8	0.1	4
9	0.5	0
10	0.5	1
11	0.5	2
12	0.5	4

Soluble principles extraction of both wild and "in vitro" plants

Plant material (1 g) such as vegetative aerial parts of wild adult plants (WAP), and micropropagated plants (MP) of 5-month-old plants (MP*) and 8-month-old plants (MP**) was dried and ground and then, macerated in 20 mL of ethanol 70° at 40 °C for 30 min in an ultrasonic bath (Ultrasonic Washer "Arcano"). The extracts were then vacuum-filtered. The filtrate fraction was dried in a rotatory evaporator and freeze-dried. The yield was determined, and the results were expressed as g of soluble principle (SP) per 100 g of dry plant material.

Determination of chemical composition

The total content of phenolic compounds was determined by using Folin–Ciocalteau reagent (Singleton & Rosi, 1965). The blue color developed was read at 765 nm in a UV/visible spectrophotometer (Jasco v-630, Thermo Fisher Scientific, Tokyo, Japan). Total flavonoids were estimated by using the Woisky and Salatino method with $AlCl_3$ reagent (Woisky & Salatino, 1998). The determinations were performed in triplicate and the results were expressed as mg of gallic acid equivalents (GAE) per gram of soluble principle (mg GAE/ g SP) and mg of quercetin equivalents (QE) per gram of soluble principle (mg QE/ g SP) for polyphenols and flavonoids, respectively.

Chalcones determination by HPLC-DAD

The HLPC-DAD system used to quantify the chalcones was a Waters 1525 equipment with a Waters 1525 binary pump system, a manual injection valve (Rheodyne Inc., Cotati, CA) with 20 µL loop, a column thermostat compartment, and a Waters 2998 diode array detector. The analysis was performed at 40 °C using a 155 x 4.6 mm XBridge™ C18 (5 µm) column with a flow rate of 0.8 $mL.min^{-1}$ (Waters Corporation, Milford, MA). The solvent system used to separate components from the extracts was composed of solvent A (0.1% acetic acid in water) and solvent B (0.1% acetic acid in methanol). The run conditions were 10% B and 90% A (0-35 min), 10 to 57% B and 90 to 43% A (35-45 min), 100% B (45-65 min). Data collection was carried out with Empower TM 2 software. The identification of phenolic compounds was performed by comparing the retention times and spectral data (220-600 nm) of each peak with those of standards (2′,4′-dihydroxychalcone and 2′,4′-dihydroxy-3-methoxychalcone) from Indofine SRL. The quantification of both chalcones was based on external calibration curves of available standards. Plots were built by comparison of area and concentration in the range of 1-500 ppm.

Biological activities of plant extracts

Antioxidant and anti-inflammatory activity

Free radical scavenging capacity by the 2,2'-azinobis-(3-ethylbenzothiazoline -6-sulfonic acid) radical cation method ($ABTS^{\bullet+}$) of extracts obtained from MP and WAP was determined according to Re et al. (1999). H_2O_2, $^{\bullet}OH$ and nitric oxide scavenging activities were determined according to Kumaran & Karunakaran (2007), Chobot (2010), and Chamira Dilanka & Preethi (2015), respectively. The results were expressed in terms of 50% free radical scavenging concentration (SC_{50}), which is defined as the concentration in soluble principle (SP) necessary to scavenge 50% of free radicals. The SC_{50} was expressed in µg $SP.mL^{-1}$.

The activity of the lipoxygenase (LOX) enzyme and xanthin-oxidase (XO) was evaluated by using spectrophotometric methods (Kong et al., 2000; Torres Carro et al., 2017). The inhibitory concentration 50 (IC_{50}) of enzyme activity was expressed in µg $SP.mL^{-1}$.

Antifungal activity

The strains were provided by the National Institute of Infectious Diseases, ANLIS "Dr. Carlos G. Malbrán" and were entered into the stock collection of the Instituto de Bioprospección y Fisiología Vegetal (INBIOFIV, CONICET-UNT). The microorganisms used were *Candida albicans* (144783; 134333), *C. glabrata* (031646; 042030), *C. tropicalis* (1841), and *Saccharomyces cerevisiae* (134528); *C. parapsilopsis* ATCC 134410 and *C. krusei* ATCC 134409 were used as reference strains. Minimal inhibitory concentration (MIC) was determined by the serial macrodilution method according to CLSI (2008). The MIC was considered as the minimum concentration of *Z. punctata* extract expressed in µg $SP.mL^{-1}$ where there was no visible growth of microorganisms after the incubation period.

Antibacterial activity

Six resistant strains of *Staphylococcus aureus* (S1, S2, S5, S6, S8, S9), isolated from patients at the Nestor Kirchner hospital were used. The MIC was determined according to CLSI (2008).

The minimum inhibitory concentration (MIC) was determined by the serial macrodilution method in agar. The MIC was considered as the minimum concentration of extract where there was no visible growth of microorganisms after the incubation period. MIC was expressed in µg $SP.mL^{-1}$.

Statistical analyses

Each experimental value is expressed as the mean ± standard deviation (SD). Statistical analysis was performed by one-way ANOVA followed by Tukey's multiple comparison test ($\alpha = 0.05$).

All statistical analyses were carried out using the Infostat software (Di Rienzo et al., 2011).

RESULTS AND DISCUSSION

Establishment of seedlings from "in vitro" seed germination

The plant material selection is a key factor for the efficacy and success of tissue culture studies (Tisserat, 1985). As the aim of the present work is the propagation for the sustainable use and conservation of *Z. punctata*, seeds are the ideal material to initiate "in vitro" cultures due to its morphogenetic potential that allows the maintenance of a wider genetic base (Fay, 1992). A 5% of seeds obtained from mature fruits belong to Group I, 65% to Group II, and only 30% to Group III. The collected seeds of Groups I and II are low GP while seeds of Group III are high GP (98%). Some authors have been reported that species with fruits as a unit of dispersal through wind, water, or animals showed a higher seed abortion than those with seeds as the unit of dispersal (Uma & Ganeshaiah, 1988). The results are in accordance with this observation. Therefore, GIII seeds were germinated under "in vitro" conditions to obtain axenic explants to introduce them into "in vitro" culture and thus achieve a greater number of plants from a reduced number of viable seeds. At least 3 explants were obtained from each seedling obtained by "in vitro" germination. In this way the number of individuals obtained from a single seed can be tripled. The seeds were cultivated in BM without and with PGRs. Both mediums showed similar established seedlings (98±2%). Those growing in the medium without the addition of PGRs showed a higher development, with an average epicotyl length of 2.37 cm longer, average of 3.31 more leaves and average of 2.19 more buds than the seedlings obtained in the medium with PGRs (Table 2). Therefore, the medium without PGRs was selected to obtain young seedlings that were used as explants for the following stage.

Shoot multiplication

The explants obtained from the geminated seedlings *in vitro* in the initial stage were cultured for induction of multiplication. Shoots of *Z. punctata* were obtained after 20 days in multiplication mediums, in all treatments with different combinations of PGRs, but the mediums 1 and 6 (Table 1) showed higher level of sprouting than the other culture medium (Fig. 3). The obtained plantlets in the treatment without PGRs (medium 1) showed a macroscopic aspect similar to plantlets produced by seeds, with highest elongation, uniform and vigorous aspect (Fig. 4 A). In mediums containing PGRs, shoots showed morphological abnormality, lack of elongation, while leaves also showed abnormalities, severe hyperhydricity symptoms, callus development and abnormal growth (Fig. 4B-E).

Anatomical studies of leaves obtained from mediums with PRGs showed a discontinues and disorganized venation patron as well as abnormal stomata with damaged cell around the stomatal pore (Fig. 5A and B). Previously, Mercado et al. (2013) described a pinnate camptodromous brochidodromous venation, poorly areolate, with a monopodial, massive, straight unbranched primary vein for the wild *Z. punctata*. Furthermore, the hyperhydric leaves obtained from mediums with PRGs have disorganized spongy and palisade-like spongy parenchyma and markedly reduced epicuticular wax compared to leaves with normal characteristics obtained in culture medium without PGRs (Fig. 5C-D). The anatomic study showed that the leaves obtained in medium without PGRs are similar to the obtained to leaves of WAP of *Z. punctata* previously described Mercado et al., 2013. For this reason, the mediums without PGRs were selected to shoot multiplication.

Although the importance of the use of auxins and cytokinins in the morphogenic response is pointed out, there are multiple examples where increasing the dose of these growth regulators causes undesirable symptoms in the development, and there are even reports of responses "in vitro"

Table 2. Average of epicotyl length, average number of leaves and average number of buds in basal culture medium (BM) and basal culture medium with plant growth regulators (BM+PGRs: BAP 0.5 mg.L^{-1} and IBA 0.1 mg.L^{-1}) and added with agar 0.75 g.L^{-1}. Parameters measured 20 days after cultivating the seeds. N = 200 in each medium. Values are reported as mean ± standard deviation of triplicates. Different letters in the same column indicate significant differences between mediums according to Tukey's test ($p \leq 0.05$).

	Epicotyl length	Leaves number	Buds Number
BM+PGRs	1.37 ± 0.32^{a}	2.51 ± 0.35^{a}	1.23 ± 0.32^{a}
BM	2.37 ± 0.38^{b}	3.31 ± 0.70^{b}	2.19 ± 0.62^{b}

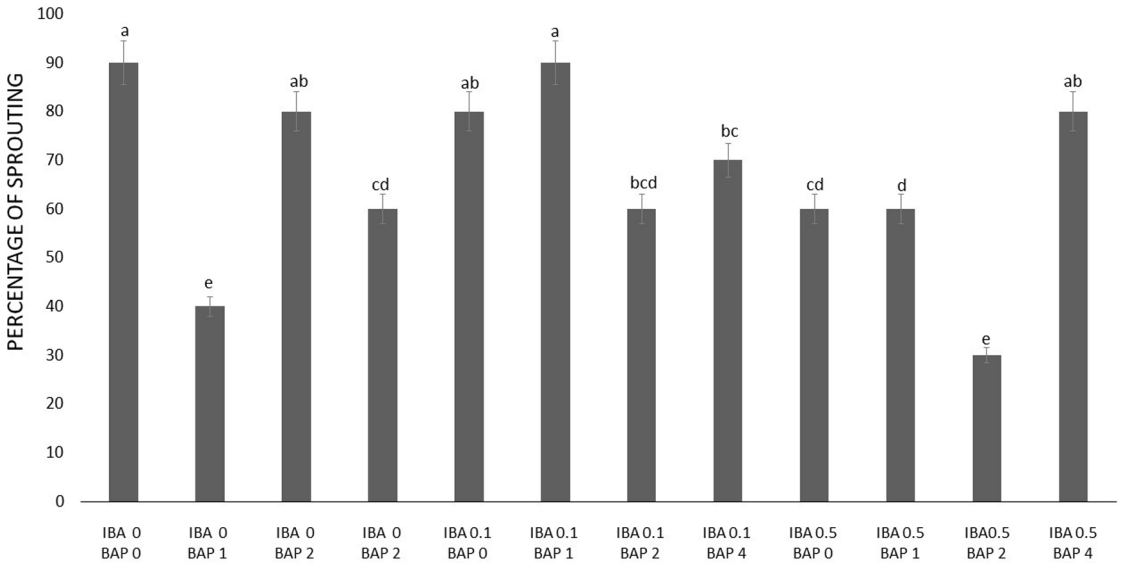

Fig. 3. Effect of different culture media on sprouting percentage. The graph shows the percentage of sprouting based on the culture media used. It indicates the IBA and BAP ratio expressed in mg.L^{-1}.

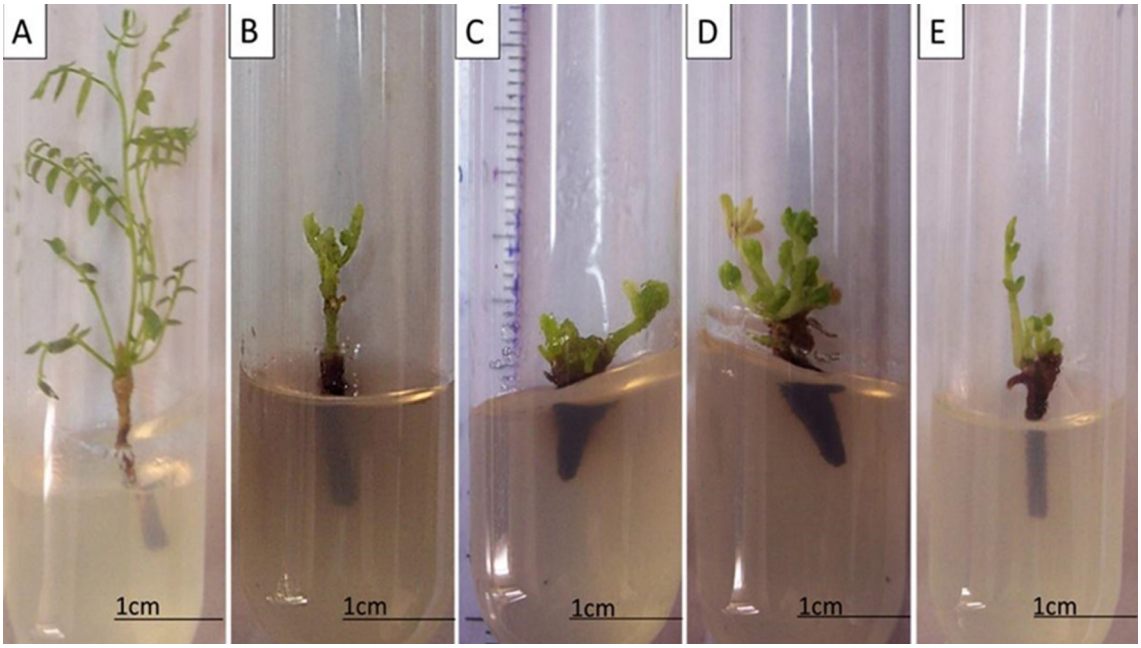

Fig. 4. Effect of different culture mediums on shoot morphology. **A.** Plantlet in culture mediums 1 without PGRs show shoots with leaves of normal appearance, similar to wild plants; **B.** Scarce elongation and leaf abnormality; **C-E.** Hyperhydricity symptoms and callus development; **D.** Chlorotic and hyperhydricity symptoms.

without the addition of regulators of exogenous growth. The latter may be due to the fact that the endogenous hormone levels were enough to ensure development (Ibrahim et al., 2008). In *Quercus suber* L., the average number of shoots per culture increased with increasing BAP concentrations up to 1 mg.L^{-1} but symptoms of abnormalities, such as shortening of internodes, compact and kinked forms, vitrification, and thickened and chlorotic leaves, were also observed (Romano et al., 1992).

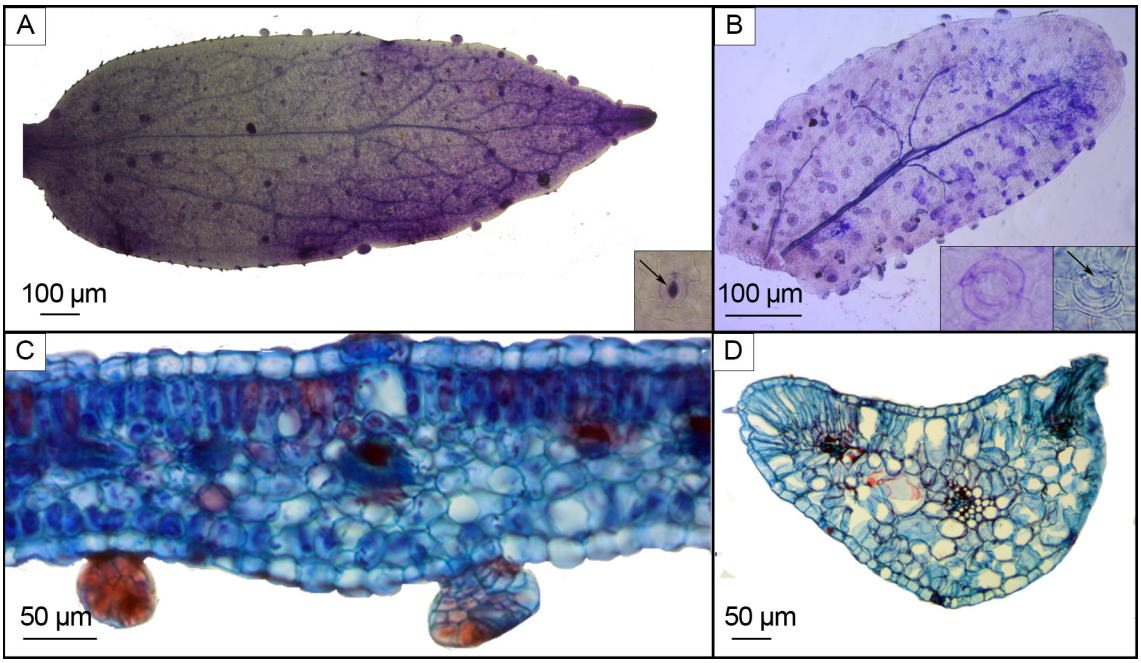

Fig. 5. A. General appearance of leaf architecture in medium without PGRs. The arrow points out the detail of a normal and functional stoma; **B.** General appearance of leaf architecture in medium with PGRs. The arrow points out the detail of a normal and non-functional stoma with a collapsed guard cell; **C.** Cross section of the lamina in shoot in medium without PGRs; **D.** Cross section of the lamina of shoot in medium with PGRs, hyperhydric leaves. The arrow points out the detail of a normal and non-functional stoma with a collapsed guard cell.

Fig. 6. Two-month-old seedlings obtained on semi-solid rooting medium from shoots of different sizes.

In melon, apple, conifers and carnation, the cytokinins induced vitrification (Dencso, 1987; Paques & Boxus, 1987; Leshem et al., 1988, Mahdiyeh et al., 2011).

Commercial growth regulators are often less effective than their endogenously produced analogs (Zaerr & Mapes, 1982). In this sense, the present results would confirm that endogenous hormones are more effective in directing morphogenic processes than synthetic growth regulators.

"In vitro" rooting

"In vitro" rooting is very often a critical phase in the woody plant propagation process. Given the aforementioned characteristics, the shoots obtained in the treatment without PGRs (medium 1) were those grown in rooting media.

The shoots developed roots (Fig. 6) in the semi-solid medium and liquid medium after two months (21.42% and 9.5%, respectively). The difference in the percentage of rooting obtained in both media was statistically significant according to the Tukey test ($p \leq 0.05$). No callus production was observed in the cervical region, which aligns with the ideal result, according to the micropropagation model by direct organogenesis (Hicks, 1987).

Rooting plants "in vitro" depends on the species. For example, *Azadirachta excelsa* L. did not root in the liquid medium (Kooi et al., 1999), whereas in woody species, the use of a liquid medium is recommended. *Thapsia garganica* L. seedlings, for example, benefited significantly from liquid rooting (Makunga et al., 2006), as was observed in other difficult-to-root species such as *Maytenus* sp. (Rathore et al., 1992) and *Cicer arietinum* L. (Jayanand et al., 2003). In the case of *Boswellia serrata* Roxb., the best rooting response was obtained when the shoots were rooted in a liquid medium (Suthar et al., 2011). However, there are woody legume species, such as *Prosopis pallida* (Humb & Bonpl. Ex Willd.) that rooting in a solid medium (Rivera Curi et al., 2020).

Acclimatization

The survival rate of acclimated *Z. punctata* plants reached 30% (two trials with 20 replicates). The percentage of surviving plants remained consistent throughout the process, including greenhouse adaptation. The acclimatized plants appeared morphologically uniform in shape, with normal leaves and 18 cm growth in a period of one year, with desirable characteristics for replanting in the natural habitat (Fig. 7).

Phytochemical characterization of micropropagated plants in comparison with wild plants

The impact on the content of secondary metabolites of the *Z. punctata* MP and greenhouse rusticated was evaluated in comparison with collected WAP in arid regions of Argentina. The content of total phenolic compounds obtained for MP of 8-month-old was higher than those obtained for WAP (Table 3). These values were higher (246.32 mg GAE.g SP^{-1}) than those previously reported for WAP (54 mg GAE.g SP^{-1}) by Zampini et al., 2012. In addition, the flavonoid content of MP was higher than the WAP. Remarkably, there were no notable qualitative distinctions in the metabolic profiles by HPLC-DAD between

Fig. 7. Acclimatized one-year-old plant in pots with a commercial substrate (Growmix).

"in vitro" cultivated and acclimatized plants with wild plants. However, the content of 2′, 4′ dihydroxychalcone (DHC), a bioactive marker metabolite of *Z. punctata* was higher in MP than WAP. The ratio between DHC/2′,4′-dihydroxy-3′-methoxychalcone (DHMC) in the plants obtained "in vitro" is approximately 1/1, while it is 1/3 in WAP (Table 3). However, this study detected and quantified, for the first time, the presence of two bioactive marker chalcones of *Z. punctata* micropropagated and acclimated plants. Considering that the chalcones are responsible of several pharmacological properties attributed to *Z. punctata* extracts, these findings are promising for ensuring a reliable and consistent source of chalcone as a potential raw material for such applications.

Biological activities of micropropagated and wild plant extracts

The extracts of vegetative aerial parts obtained "in vitro" of 5-month-old plants and of 8-month-old plants, and the extracts of WAP showed antioxidant capacity with similar antioxidant potency (Table 4). They also showed capacity to inhibit the enzyme lipooxygenase (LOX) and xanthine oxidase (XO), two remarkable enzymes in inflammatory processes (Table 4).

When comparing the IC_{50} values of the different extracts on enzyme of inflammatory process, it was observed that *Z. punctata* plants obtained by micropropagation and kept for 5 months in greenhouses have a higher inhibitory potency against both enzymes than the extracts obtained from *Z. punctata* wild adult plants (Table 4).

The extracts corresponding to MP and WAP exhibited similar antibacterial and antifungal activity. They showed efficacy against the yeast species and were able to inhibit the growth of antibiotic-resistant *Staphylococcus aureus* strains with similar potency (Tables 5, 6).

Table 3. Yield, total phenolic compounds, flavonoids and chalcones of plant material obtained by micropropagation "in vitro" and wild adult plants collected in Monte desert region.

	Yield (g SP/100 g PM)	Total phenolic compounds (mg GAE/ g SP)	Flavonoid content (mg QE. g^{-1} SP)	µgDHC/mg SP	µgDHMC/mg SP
WAP	54.00 ± 0.10[a]	229.83 ± 4.50[a]	47.26 ± 1.33[a]	10.6 ± 0.10[a]	33.9 ± 0.33[a]
MP*	51.38 ± 0.10[a]	234.60 ± 6.81[a]	66.34 ± 1.77[b]	29.2 ± 1.00[b]	26.0 ± 0.10[a]
MP**	57.50 ± 0.10[a]	246.32 ± 5.20[b]	66.40 ± 1.30[b]	23.2 ± 0.33[b]	29.2 ± 0.20[a]

GAE: gallic acid equivalent, QE: quercetin equivalent, SP: soluble principle, PM: Dry Plant Material
DHC: 2′, 4′- dihydroxychalcone y DHMC: 2′, 4′-dihydroxi-3′-methoxychalcone
WAP: wild adult plant
MP*: micropropagated plant (5-month-old plants)
MP** micropropagated plant (8-month-old plants)
Equal letters in each column indicate no statistically significant difference according to Tukey's test ($p \leq 0.05$))

Table 4. Antioxidant and antiinflammatory activities of hydroalcoholic extracts of plants obtained by micropropagation "in vitro" and wild adult plants collected in Monte desert region.

Samples	$ABTS^{+\cdot}$ Scavenging	H_2O_2 Scavenging	$^{\cdot}OH$ Scavenging	XO Inhibition	LOX Inhibition
	SC_{50} (µg SP.mL^{-1})			IC_{50} (µg SP.mL^{-1})	
WAP	9.90 ± 0.50[a]	66.0 ± 1.04[a]	0.48 ± 0[a]	26.97 ± 2.10[b]	65.68 ± 2.20[b]
MP*	9.01 ± 1.20[a]	62.9 ± 2.32[a]	0.47 ± 0.01[a]	25.50 ± 2.30[b]	66.44 ± 1.40[b]
MP**	9.35 ± 0.85[a]	63.10 ± 1.50[a]	0.39 ± 0.04[a]	19.15 ± 1.50[a]	54.38 ± 2.10[a]

$^{\cdot}OH$ Hydroxyl radical; LOX: lipoxygenase enzyme; XO: xanthin-oxidase. WAP: vegetative aerial parts of wild adult plant
WAP: wild adult plant
MP*: micropropagated plant (5-month-old plants)
MP** micropropagated plant obtained "in vitro" (8-month-old plants)
$SC_{50;}$ Scavenging concentration of 50% free radical
$IC_{50;}$ Inhibitory concentration of 50% enzyme activity
SP: Soluble principle
Equal letters in each column indicate no statistically significant difference according to Tukey's test ($p \leq 0.05$)

Table 5. Antifungal activity of hydroalcoholic extracts of plants obtained by micropropagation "in vitro" and wild adult plants collected in Monte desert region.

Strain	Phenotypes of clinical isolates	MIC (µg SP.mL^{-1}) WAP	MIC (µg SP.mL^{-1}) MP*	MIC (µg SP.mL^{-1}) MP**
144783	Flu^S,Am^S ,Ny^S	400	800	800
134333	Flu^R,Am^S ,Ny^S	800	400	400
031646	Flu^S,Am^S ,Ny^S	800	800	800
1841	Flu^S,Am^S Ny^S	400	400	400
134528	Flu^S,Am^S Ny^S	400	400	400
ATCC-134410	Flu^S,Am^S Ny^S	400	400	400
ATCC-134409	Flu^R,Am^S Ny^S	400	800	800

Candida albicans 144783 and 134333, *Candida glabrata* 031646, *Candida tropicalis* 1841, *Sacharomyces cerevisiae* 134528, *Candida parapsilosis* ATCC-134410, *Candida krusei* ATCC-134409. Flu: Fluconazole; Am: Amphotericin B; Ny: Nystatin; R: Resistant; S: Sensitive. MIC: minimal inhibitory concentration. SP: Soluble principle
WAP: wild adult plant
MP*: micropropagated plant (5-month-old plants)
MP** micropropagated plant obtained "in vitro" (8-month-old plants)

Table 6. Antibacterial activity of hydroalcoholic extracts of plants obtained by micropropagation "in vitro" and wild adult plants collected in Monte desert region.

Strain	Phenotypes of clinical isolates	MIC (µg SP.mL^{-1}) WAP	MIC (µg SP.mL^{-1}) MP*	MIC (µg SP.mL^{-1}) MP**
S1	$Oxa^R Gen^R Eri^R$	800	800	800
S2	$Oxa^R Gen^R$	800	800	800
S5	$Oxa^R Gen^R Cip^R Lev^R Mox^R$	800	800	800
S6	$Oxa^R Eri^R$	800	800	800
S8	$Oxa^R Gen^R$	800	800	800
S9	$Oxa^R Gen^R Cip^R Lev^R Mox^R Eri^R Cli^R$	800	800	800
ATCC-29213		800	800	800
ATCC-43300		800	800	800

Staphylococcus aureus strains, ATCC29213, ATCC43300, R: Resistant, Oxa: Oxacycline; Gene: Gentamicin; Cip: Ciprofloxacin; Lev: Levofloxacin; Mox: Moxifloxacin; Eri: Erythromycin; Cli: Clindamycin
WAP: wild adult plant
MP*: micropropagated plant (5-month-old plants)
MP** micropropagated plant obtained "in vitro" (8-month-old plants)

CONCLUSIONS

Whole healthy plants were obtained by "in vitro" culture without growth regulators throughout the period ranging from the establishment of explants up to the multiplication of shoots. The development described in the present work could serve as a starting point for optimizing the mass production of *Z. punctata*, as well as other species, that grow in similar desert environments. In addition, the micropropagation would allow controlled growth independent of seasonal and climatic variations. The re-implantation of *Zuccagnia punctata* in its natural habitat in northwestern Argentina could prevent the loss of this genetic resource and promote its sustainable use. No significant changes in the yield of soluble principle and phenolic content were observed in micropropagated plant and wild adult plant but the flavonoid content was higher in micropropagated plant than wild adult plant. For the first time, 2′, 4′ dihydroxychalcone, the main bioactive of *Z. punctata* was identified and quantified in the ethanolic extract from micropropagated plant. Regardless of the biological activities, the ethanolic extracts of *Z. punctata* obtained by "in vitro" culture presented high activity against both Gram-positive and *Candida* and maintain its antioxidant and anti-inflammatory capacity. This successful initiation of "in vitro" cultures ensure a reliable and consistent source of *Z. punctata* as a potential raw material for pharmacological applications.

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