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ORIGINAL ARTICLE

Establishment of callus-cultures of the Argentinean mistletoe, *Ligaria cuneifolia* (R. et P.) Tiegh (Loranthaceae) and screening of their polyphenolic content

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Abstract

Ligaria cuneifolia (R. et P.) Tiegh (Loranthaceae), known as *liga, muérdago criollo*, or Argentinean mistletoe, is a hemiparasitic plant with a broad distribution in central and northern Argentina. Pharmacological studies showed that *L. cuneifolia* extracts have hypolipemic, antioxidant, antibacterial, and immunomodulatory effects. We have established callus cultures from embryo and haustoria fragments. The highest frequency of callus formation from embryos (85%) was obtained on White medium with 4% (w/v) sucrose and 2.5 µM 1-naphtalene acetic acid and 9.2 µM kinetin as plant growth regulators (PGRs). From haustoria, the best result (35%) was obtained on Gamborg medium with 3% (w/v) sucrose and 0.45 µM 2,4-dichlorephenoxyacetic acid and 0.47 µM zeatin as PGRs. Thin layer chromatography showed that callus methanolic extract (2.5% w/v) had a lower content of flavonoids and proanthocyanins as compared to the wild plant (5% w/v for leaves, stems, and flowers), but a higher content of hydroxycinnamic acids. High performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS) showed the presence of quercetin glycosides and phenolic acids in the methanolic extracts both from the parent plant and the callus obtained from embryo.

Key message

Callus cultures were established from embryo and haustorium explants of *Ligaria cuneifolia*. Leaves, stems, and meristems were recalcitrant to in vitro culture. Callus tissues contained quercetin glycosides and phenolic acids.

Keywords Medicinal plants · *Liga* · Hemiparasitic plant · *Callus* culture · Flavonoids

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Introduction

Ligaria cuneifolia (R. et P.) Tiegh (Loranthaceae), known as *liga, muérdago criollo*, or Argentinean mistletoe, is an aerial-photosynthesizing hemiparasitic plant widely distributed in Latin America, from Perú to Central Argentina (Abiatti 1946; Amico and Nickrent 2007). Its hosts are mostly members of the Fabaceae family such as *Prosopis caldenia* Burkart P*, P. torquata* (Lag.) DC*, Geoffroea decorticans* (Hook & Arn.), and *Acacia caven* (Molina) Molina. Also, it can be found growing on species from the Anacardiaceae (e.g. *Schinus fasciculata* I.M. Johnst., *S. areira* L.), Ulmaceae (e.g. *Ulmus pumila* L.), and Verbenaceae [e.g.: *Aloysia gratisima* (Gillies & Hook) Tronc.] families. *L. cuneifolia* flowers are hexamer-shaped, arranged in clusters, and colored from orange to red, according to the region where they grow. Fruits are dark violet or black berries that are dispersed by birds (Varela et al. 2001; Amuchástegui et al. 2003). It has a haustorium (a modified root structure) that extracts water and minerals from the host xylem producing a vascular continuity (Rustán et al. 2003).

Preparations of *L. cuneifolia* are widely used in popular (folk and indigenous) medicine in the northern and central provinces of Argentina for its attributed therapeutic properties (Scarpa and Montani 2011). The infusion prepared from the aerial parts is taken as a substitute of *Viscum album* L. (Santalaceae) in cases of high blood pressure (Martínez 2010). In addition, the infusion or the decoctions are used as antihemorrhagic, abortive, emmenagogue, oxytocic, in case of fractures, and sore throat. Also, it is used in veterinary and as forage for goats and cattle (Scarpa and Montani 2011).

Pharmacological studies support the use of *L. cuneifolia* in popular medicine as antioxidant, antibacterial and as anti-hypertensive or anti-hypotensive depending on the host (Soberón et al. 2014; Gonzálvez et al. 2017). It was also reported the inhibition of the proliferation of murine mitogen-activated lymphocytes, murine T cell leukemia (LB) and breast tumor cells (MMT) by *L. cuneifolia* whole extract and the ethyl acetate flavonoid fraction (Cerdá Zolezzi et al. 2005).

Some of the pharmacological activities of *L. cuneifolia* are attributed to its content in lectins and polyphenolic compounds. The main identified polyphenolic compounds in *L. cuneifolia* extracts are quercetin-3-O-glycosides, such as quercetin-3-O-glucoside, quercetin-3-O-xyloside, quercetin-3-O-arabinopyranoside, quercetin-3-O-arabinofuranoside, quercetin-3-O-rhamnoside, and four novel quercetin-galloyl-glycosides: quercetin-3-O-(2″-O-galloyl) rhamnoside, quercetin-3-O-(3″-O-galloyl) rhamnoside, quercetin-3-O-(2″galloyl)-arabinofuranoside, and quercetin-3-O-(2″-O-galloyl)-arabinopyranoside. Catechin, the main flavan-3-ol, and other proanthocyanidins with different degree of polymerization were also identified (Varela et al. 2001; Dobrecky et al. 2017). Depending on the geographical area, the presence of tyramine was shown (Vázquez y Novo et al. 1989).

L. cuneifolia cannot be cultured in the field; moreover, its exploitation in order to extract the active metabolites could imply a threat to the conservation of the species. In this context, in vitro cultures appear as an attractive alternative production platform (Espinosa-Leal et al. 2018). Studies on in vitro cultures are scarce in the Loranthaceae family. In the case of *L. cuneifolia* they are non-existent, possibly due to the difficulty in culturing a hemiparasitic plant and because of the explant exudation of phenolics leading to medium browning and necrosis (Ishrad et al. 2018). The aim of this work was the establishment of callus cultures of *L. cuneifolia* and the screening of their polyphenolic content.

Materials and methods

Plant material

Plant material was collected between January 2015 and December 2017 from the region (30 km) comprised between La Población, Córdoba (32°03′34.4″S 65°00′36.6″W) and Villa de Merlo, San Luis (32°21′22.5″S 65°00′20.5″W), Argentina. Samples were placed in plastic bags and stored at 4 °C until being processed. A voucher was deposited in the *Museo de Farmacobotánica Juan Aníbal Dominguez* herbarium (Buenos Aires, Argentina) BAF 9018.

Chemicals and reagents

Gamborg et al. (1968), White (1963), and Murashige and Skoog (1962) media, sucrose, casein hydrolysate and agar (plant tissue micropropagation culture grade) were from PhytoTechnology Laboratories (Lenexa, KS). LiChrosolv® Methanol was supplied from Merck (Darmstadt, Germany). Formic acid was purchased from Baker (New Jersey, USA). Ultrapure water was generated with a Barnstead Thermo Scientific™ (Waltham, Massachussets). Quercetin-3-rhamnoside (Q-3-O-Rh) was from Extrasynthese (Lyon, France); catechin (C), quercetin-3-O-glucoside (Q-3-O-G), quercetin-3-O-xyloside (Q-3-O-X), quercetin-3-O-arabinofuranoside (Q-3-O-AF), quercetin-3-O-arabinopyranoside (Q-3-O-AP) and chlorogenic acid (CA) were from Sigma (St. Louis, MO, USA). The other chemical, standards, and solvents were purchased from Sigma-Aldrich® (Saint Louis, MO).

Initiation of in vitro callus cultures

Surface sterilization

Plant material was washed with tap water to remove dust, insects, etc. Then, it was immersed in a 0.2% v/v Tween 20 solution for 60 s and stirred. Young leaves, stems and meristems were dipped in a sodium hypochlorite solution (NaClO, 4% active chloride) for 15 min or in mercuric chloride ($HgCl_2$) at different concentrations (0.05, 0.1, or 0.2% w/v) with a previous treatment with ethanol (70%) (Majid et al. 2014) during 30 or 60 s. Fruits were sterilized with NaClO as described above and used to obtain seeds and embryos. In all cases, after the treatment, explants were washed three times with sterile distilled water under laminar flow cabinet.

Oxidative browning removal

As leaves, meristems, and stems produced browning exudation when cultured in vitro in MSRT medium (Nigra et al. 1987), different procedures were performed in order to avoid it. A first experiment was made culturing the $HgCl_2$-sterilized explants in minimal White medium or White medium with the addition of activated charcoal and polyvinylpyrrolidone (PVP) (Trigiano 2011). A second experiment involved soaking the explants in filter sterilized (0.22 µm Millipore) antioxidant solutions (citric acid, ascorbic acid, L-cysteine HCl, $AgNO_3$) at different concentrations during 5, 15, or 30 min after sterilization with $HgCl_2$ (on line resource 1) (Karunaratne et al. 2014; Ndakidemi et al. 2014; Ahmad et al. 2016). Then, explants were transferred to sterile Petri dishes containing White medium supplemented with 8 g/L agar and 4.5 µM 2,4-dichlorophenoxyacetic acid (2,4-D), pH 5.6–5.8. The test was performed in complete darkness or under a 16-h light photoperiod as described below. A third experiment was performed subculturing the explants to fresh media for five times daily as was described by other authors (Ahmad et al. 2013). Presence or absence of oxidative browning exudation was visually assessed.

Influence of explant source, culture media, and PGRs

For callus induction, 1 cm^2-young leaf fragments, 1 cm-length stem pieces, or 5 mm meristem pieces were separated from the whole plant. To obtain embryos and haustoria, the exocarp was removed from the fruits. Embryos were separated from the naked fruit with a scalpel under a laminar flow cabinet. As for haustorium, they developed after transferring the seeds to culture vessels with half strength MS medium with RT vitamins (Khana and Staba 1968), 8 g/L agar and 3% w/v sucrose, pH 5.6–5.8. After 15 days in culture, 1 cm-long pieces from the elongated haustoria were taken for callus initiation.

In the case of leaves, stems, and meristems explants, after the sterilization and pre-treatment with the most effective antioxidant solution, they were transferred to glass tubes containing approximately 10 mL of culture medium. The culture medias assayed were MSRT, half-strength MSRT, and White all at pH 5.6–5.8, and Gamborg B5 at pH 5.5. All media included 8 g/L of agar and 30 g/L sucrose. PGRs were added as detailed in Table 1.

In the case of embryos, after sterilization they were transferred to glass tubes with 10 mL of White medium with the addition of 500 mg/L casein hydrolysate (Johri and Bajaj 1962, 1964; Bajaj 1967; Ohofeghara 1971). The sucrose concentration and PGRs relationships were set according to a full factorial design. The pH was adjusted to 5.6–5.8. Four central points were used to analyze the effect of sucrose, 1-naphtalene acetic acid (NAA) and kinetin (Kin) concentrations on the initiation of in vitro cultures from embryos. This particular design was chosen to assess a possible third order interaction between the variables. The central points were included to evaluate the curvature of the resulting model. The response variable was the percentage of callus formation. Each experimental unit was composed of 10 embryos and was run twice. The 20 randomized runs where divided into two blocks, each of 10 runs, to split the experiment into 2 days due to the high workload (Table 2). The results were

Table 1 Plant growth relationships used for initiating *Ligaria cuneifolia* in vitro cultures from leaves, stems, and meristems

Treatment	Auxin (µM)	Cytokinin (µM)
1	2,4-D 2.25	–
2	2,4-D 4.50	–
3	2,4-D 10.0	–
4	IAA 2.25	–
5	IAA 4.50	–
6	IAA 10.0	-
7	2,4-D 2.25	Kin 0.46
8	2,4-D 2.25	Kin 2.30
9	2,4-D 4.50	Kin 0.46
10	2,4-D 4.50	Kin 2.30
11	2,4-D 10.0	Kin 0.46
12	2,4-D 10.0	Kin 2.30
13	NAA 5.40	Kin 0.46

All treatments were made with n = 10 in MSRT, MS/2, Gamborg B5 or White culture media, in a growth chamber at 24 ± 2 °C, a 16-h light photoperiod with an irradiance 1.8 w/m^2/s

2,4-D 2,4-dichlorophenoxyacetic acid, *NAA* 1-naphthaleneacetic acid, *IAA* indole-3-acetic acid, *KIN* kinetin

Table 2 2^3 full factorial design matrix used for the in vitro cultures initiation from *Ligaria cuneifolia* embryo

Standard order	Block	Run order	Factor 1 A:sucrose	Factor 2 B:NAA	Factor 3 C:kin	Response 1 Callus formation
			% v/v	µM	µM	%
1	Day 1	9	2	2.50	2.30	0
2	Day 2	17	2	2.50	2.30	20
3	Day 1	3	4	2.50	2.30	60
4	Day 2	19	4	2.50	2.30	66
5	Day 1	10	2	10.70	2.30	40
6	Day 2	13	2	10.70	2.30	55
7	Day 1	5	4	10.70	2.30	60
8	Day 2	15	4	10.70	2.30	30
9	Day 1	6	2	2.50	9.20	55
10	Day 2	14	2	2.50	9.20	90
11	Day 1	2	4	2.50	9.20	100
12	Day 2	12	4	2.50	9.20	70
13	Day 1	4	2	10.70	9.20	20
14	Day 2	11	2	10.70	9.20	20
15	Day 1	8	4	10.70	9.20	60
16	Day 2	20	4	10.70	9.20	60
17	Day 1	1	3	6.60	5.75	88
18	Day 1	7	3	6.60	5.75	40
19	Day 2	16	3	6.60	5.75	80
20	Day 2	18	3	6.60	5.75	66

All cultures were performed on basal White medium with casein hydrolysate (500 mg/L), pH 5.6–5.8, in a growth chamber at 24 ± 2 °C, a 16-h light photoperiod and an irradiance 1.8 w/m^2/s

NAA 1-naphthaleneacetic acid, *Kin* kinetin

analyzed using the software Design Expert 11 Trial version (Stat-Ease and 2018). Subcultures were performed every 4 weeks.

In the case of haustoria, explants were directly transferred to tubes containing 10 mL Gamborg media with 3% w/v sucrose and PGRs at different relationships. The PGRs tested were 2,4-D, NAA, indole-3- acetic acid (IAA), 6-benzylaminopurine (BAP), indole butyric acid (IBA), zeatin, and Kin. The pH was adjusted to 5.5. The results were analyzed through a contingency table, a chi square test of independence and the Fisher's Exact Test for Count Data (Table 3). This last test was used since more than 20% of the contingency table cells had expected count values lower than five (McHugh 2013). The chi squared was calculated using the function *chisq.test* and the Fisher's Exact Test using the function *fisher.test*, both from the R base *stats* package in the R studio software (R Core Team 2018; RStudio Team 2018). Post hoc analysis was done using the adjusted standardized residuals (*Adj Res*) method following the recommendations of Sharpe (2015). The 0.05 critical value (α) and its z critical = ± 1.96 were corrected using the Bonferroni method, as recommended in the case of large contingency tables by Sharpe (2015), resulting in α = 0.000757 and a z = ± 3.37. Subcultures were performed every 4 weeks.

Growth index (GI): it was calculated as the ratio of the final fresh weight (FW) to the initial FW (Payne et al. 1991).

Culture conditions: all the experiments were performed in a growing chamber at 24 ± 2 °C, under a 16-h light photoperiod using fluorescent daylight lamps (Narva T8 LT 18 W/760-010 daylight, Germany) with an irradiance intensity of 13.5 µmol/m^2/s. In the case of oxidative browning removal, a set of the experiment was also performed in continuous darkness.

Qualitative and quantitative analysis of polyphenolic compounds

Thin layer chromatography (TLC): methanolic extracts from leaves, stems, and flowers (5% w/v) and calli

Table 3 Contingency table and chi square test of independence results for the experiment on the effect of plant growth regulators on the initiation of callus from *L. cuneifolia* haustoria

Treatment		Callus viability		Marginals	Plant growth regulator
		Yes	No		
1	Observed	0	49	49	2,4-D (2.25 µM)
	Expected	3.28	45.7		
	Adj Res	− 1.95	1.96		
2	Observed	1	17	18	2.4-D (4.5 µM)
	Expected	1.21	16.8		
	Adj Res	− 0.19	0.05		
3	Observed	0	20	20	2.4-D (10 µM)
	Expected	1.34	18.7		
	Adj Res	− 1.18	0.31		
4	Observed	1	19	20	2.4-D + KIN (2.25 µM:2.3 µM)
	Expected	1.34	18.7		
	Adj Res	− 0.30	0.07		
5	Observed	0	18	18	2,4-D + KIN (4.5 µM:2.3 µM)
	Expected	1.21	16.8		
	Adj Res	− 1.12	0.30		
6	Observed	0	20	20	2,4-D + KIN (10 µM:2.3 µM)
	Expected	1.34	18.7		
	Adj Res	− 1.18	0.31		
7	Observed	1	19	20	2,4-D + KIN (2.25 µM:4.65 µM)
	Expected	1.34	18.7		
	Adj Res	− 0.30	0.07		
8	Observed	0	19	19	2,4-D + KIN (4.5 µM:4.65 µM)
	Expected	1.27	17.7		
	Adj Res	− 1.14	0.31		
9	Observed	0	19	19	2,4-D + KIN (10 µM:4.65 µM)
	Expected	1.27	17.7		
	Adj Res	− 1.14	0.31		
10	Observed	0	19	19	2,4-D + KIN (0.45 µM:0.46 µM)
	Expected	1.27	17.7		
	Adj Res	− 1.14	0.31		
11	Observed	1	17	18	IAA (2.25 µM)
	Expected	1.21	16.8		
	Adj Res	− 0.19	0.05		
12	Observed	3	17	20	IAA (4.5 µM)
	Expected	1.34	18.7		
	Adj Res	1.46	− 0.40		
13	Observed	2	18	20	IAA (10 µM)
	Expected	1.34	18.7		
	Adj Res	0.58	− 0.16		
14	Observed	4	16	20	NAA + KIN (2.25 µM:2.30 µM)
	Expected	1.34	18.7		
	Adj Res	2.33	− 0.63		
15	Observed	4	16	20	NAA + KIN (4.5 µM:2.30 µM)
	Expected	1.34	18.7		
	Adj Res	2.33	− 0.63		

Table 3 (continued)

Treatment		Callus viability		Marginals	Plant growth regulator
		Yes	No		
16	Observed	2	17	19	NAA + KIN (10 µM:2.30 µM)
	Expected	1.27	17.7		
	Adj Res	0.66	−0.17		
17	Observed	4	15	19	NAA + KIN (0.45 µM:0.46 µM)
	Expected	1.27	17.7		
	Adj Res	2.46	−0.65		
18	Observed	0	19	19	BAP (0.25 µM)
	Expected	1.27	17.7		
	Adj Res	−1.14	0.31		
19	Observed	1	17	18	BAP (1 µM)
	Expected	1.21	16.8		
	Adj Res	−0.19	0.05		
20	Observed	1	18	19	BAP (0.5 µM)
	Expected	1.27	17.7		
	Adj Res	−0.24	0.07		
21	Observed	0	17	17	2,4-D + zeatin (2.25 µM:0.23 µM)
	Expected	1.14	15.9		
	Adj Res	−1.08	0.28		
22	Observed	0	18	18	2,4-D + zeatin (4.5 µM:0.23 µM)
	Expected	1.21	16.8		
	Adj Res	−1.12	0.30		
23	Observed	0	19	19	2,4-D + zeatin (10 µM:0.23 µM)
	Expected	1.27	17.7		
	Adj Res	−1.14	0.31		
24	Observed	1	15	16	2,4-D + zeatin (2.25 µM:0.47 µM)
	Expected	1.07	14.9		
	Adj Res	−0.07	0.03		
25	Observed	1	16	17	2,4-D + zeatin (4.5 µM:0.47 µM)
	Expected	1.14	15.9		
	Adj Res	−0.13	0.03		
26	Observed	2	18	20	2,4-D + zeatin (10 µM:0.47 µM)
	Expected	1.34	18.7		
	Adj Res	0.58	−0.16		
27	Observed	7	13	20	2,4-D + zeatin (0.45 µM:0.47 µM)
	Expected	1.34	18.7		
	Adj Res	**4.97**	−1.34		
28	Observed	4	14	18	IBA + BAP (4.90:0.5 µM)
	Expected	1.21	16.8		
	Adj Res	2.57	−0.69		
29	Observed	1	19	20	IBA + BAP (4.90:0.4 µM)
	Expected	1.34	18.7		
	Adj Res	−0.30	0.07		
30	Observed	2	18	20	IBA + BAP (4.90:0.2 µM)
	Expected	1.34	18.7		
	Adj Res	0.58	−0.16		
31	Observed	1	18	19	2,4-D + IAA (2.25:2.25)
	Expected	1.27	17.7		
	Adj Res	−0.24	0.07		

Table 3 (continued)

Treatment		Callus viability		Marginals	Plant growth regulator
		Yes	No		
32	Observed	0	20	20	2,4-D + IAA (4.5:4.5)
	Expected	1.34	18.7		
	Adj Res	− 1.18	0.31		
33	Observed	0	19	19	2,4-D + IAA (0.46:0.46)
	Expected	1.27	17.7		
	Adj Res	− 1.14	0.31		
Marginals		44	613	657	

Statistics			
Chi square test of independence results			
	Value	df	*p*
X^2	74.225	32	0.00003325
N	657		
Fisher's exact test for count data			
$p = 0.0000007039$			

The highlighted value, treatment 27, corresponds to an adjusted standardized residual (Adj Res) superior than 3.37. Cultures were performed in Gamborg media with 3% w/v sucrose, pH 5.5, in a growth chamber at 24 ± 2 °C, a 16-h light photoperiod and an irradiance of 1.8 w/m^2/s

2,4-D 2,4-dichlorophenoxyacetic acid, *NAA* 1-naphthaleneacetic acid, *IAA* indole-3- acetic acid, *BAP* 6-benzylaminopurine, *IBA* indole butyric acid, zeatin, *Kin* kinetin

initiated from embryo (2.5% w/v) were made. Plant material was previously air-dried in darkness (25 °C for 7 days). The qualitative analysis of polyphenols by TLC was made with Silica Gel 60 (Merck) as stationary phase and ethyl acetate: formic acid: acetic acid: water (100:11:11:23) as mobile phase. Flavonoids and hydroxycinnamic acid derivatives were revealed with a 1% v/v methanolic solution of the reagent for natural products (NP, AEDBE: 2-aminoethyl diphenyl borate ester, Sigma-Aldrich), and proanthocyanidins with an ethanolic solution of vainillin:HCl (4:1).

High performance liquid chromatography-tandem mass spectrometry (HPLC MS/MS): dried callus and parental plant material obtained as described above were successively extracted with pure methanol (5% w/v) and combinations of 80% methanol–water and 50% methanol–water with continuous shaking. Then, they were evaporated to dryness under reduced pressure in a rotary evaporator and further resuspended in methanol, filtered and finally diluted 1/5 in 0.1% aq. formic acid. HPLC–MS/MS system consisted of UltiMate 3000 HPLC coupled to a TSQ Quantum Access MAX Triple Quadrupole Mass Spectrometer with electrospray ionization (Massachusetts). Positive mode was employed for quercetin glycosides and negative mode for chlorogenic acid. The column was a C18 Hypersil 150 × 4 mm, 5 µm particle size Thermo Scientific™. HPLC conditions: The mobile phase consisted of a gradient of 0.1% formic acid in methanol and 0.1% aqueous formic acid 15:85 (0 min), 40:60 (25 min), 40:60 (50 min), 85:15 (60 min), return to starting conditions and equilibration in 15 min. Flow rate was 1 mL/min and the temperature of the oven and sampler were set at 40 °C and 20 °C respectively. Injection volume was 25 µL MS/MS conditions: Tuning conditions were as follows: spray voltage 3.5 kV, vaporizer temperature 233 °C, capillary temperature 314 °C, while sheath gas pressure and aux gas pressure were set at 10 and 45 units respectively. The instrument method comprised two scan events involving single ion monitoring (SIM) and single reaction monitoring (SRM). Mix standard solution: Stock standard solutions of 1 mg/mL were prepared in methanol. A mix standard solution was prepared by combining aliquots of each stock standard

solution and diluting with 0.1% aq. formic acid to a final concentration of 1 µg/mL.

Results and discussion

Initiation of in vitro cultures

Surface sterilization

As for leaves, stems, and meristems the most efficient treatment (80% sterility) was obtained by dipping the explants in alcohol 70% (30 s exposure) followed by a treatment with $HgCl_2$ (indistinctively 0.05 or 0.1% w/v). In the case of seeds and embryos the efficiency of sterilization was 85% and 92.4% respectively.

Oxidative browning removal

Explants from leaves, meristems, and stems in MSRT medium produced browning exudation within a few hours resulting toxic and leading to the death of the tissues in a few days, as was reported previously with other species (Debergh and Read 1991). The same was observed in explants cultured in White medium or White medium with adsorbing agents (activated charcoal and PVP). Sub-culturing the explants every 24 h to fresh medium strongly reduced oxidative browning from the third transfer, although it resulted a cumbersome procedure (time and labor consuming). Finally, we have succeeded in inhibiting browning by soaking the explants in different antioxidant solutions (online resource 1) either in darkness or under a 160-h light photoperiod,

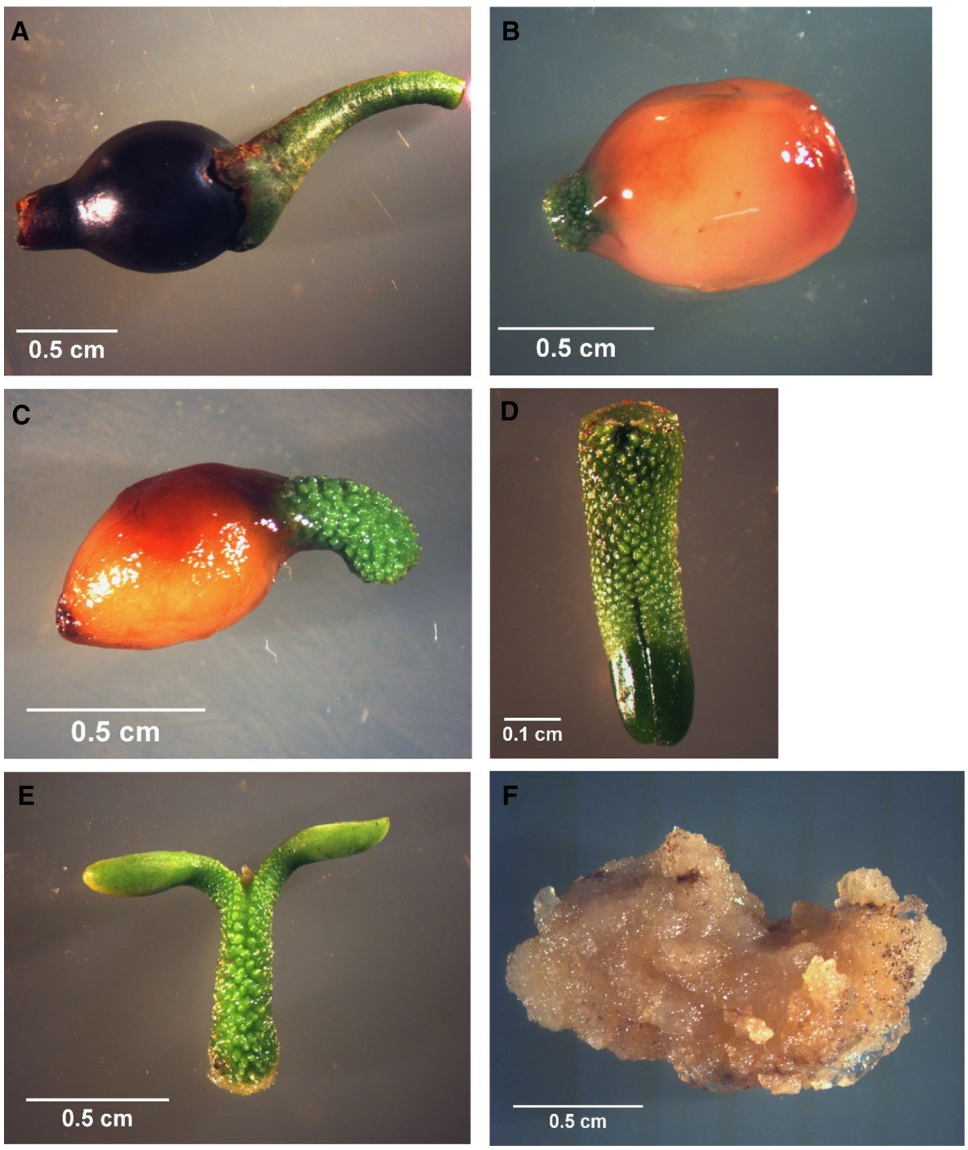

Fig. 1 *Ligaria cuneifolia* explants and callus. **a** Fruit with pedicel. **b** Seed in half strength MS medium with RT vitamins and 3% w/v sucrose, day 0. **c** 3 day developed haustorium in B5 medium with 2,4-D (2.25 µM). **d** Embryo in White medium, day 0. **e** Embryo in White medium with casein hydrolysate 500 mg/L, NAA KIN (2.70 µM:9.20 µM), and sucrose 4% (w/v), day 9. **f** Callus developed from embryo in White medium with casein hydrolysate 500 mg/L, ANA: Kin (2.70 µM:9.20 µM), sucrose 4% (w/v), 3 months in culture. Cultures are developed in a growth chamber at 24 ± 2 °C, 16-h light photoperiod, and 13.5 µmol/m^2/s irradiance

Table 4 ANOVA table for in vitro cultures initiation from *Ligaria cuneifolia* embryo

Source	Sum of squares	df	Mean square	F-value	*p*-value	
Block	57.80	1	57.80			
Model	9060.75	7	1294.39	4.150	0.021	Significant
A-sucrose	2652.25	1	2652.25	8.510	0.015	Significant
B-NAA	841.00	1	841.00	2.700	0.132	
C-Kin	1296.00	1	1296.00	4.160	0.069	
AB	196.00	1	196.00	0.629	0.446	
AC	1.00	1	1.00	0.003	0.956	
BC	2352.25	1	2352.25	7.550	0.021	Significant
ABC	1722.25	1	1722.25	5.530	0.041	Significant
Curvature	1051.25	1	1051.25	3.370	0.096	
Residual	3116.20	10	311.62			
Lack of fit	1866.20	8	233.27	0.3730	0.871	Non-significant
Pure error	1250.00	2	625.00			
Cor total	13286.00	19				

A sucrose, *B* 1-naphthaleneacetic acid (*NAA*) and *C* kinetin (*Kin*). AB. AC and BC represent second order interactions between the variables. ABC represents a triple order interaction. *p* values lower than 0.05 were considered significant

Table 5 In vitro culture initiation from *Ligaria cuneifolia* embryo expected values if the optimal conditions for each variable are used

Response	Predicted mean	Std dev	SE mean	95% CI low for mean	95% CI high for mean
Callus formation (%)	85	17.6	12.3	57.5	112.5

In this case sucrose = 4% w/v, 1-naphthaleneacetic acid (*NAA*) = 2.50 µM and kinetin (*Kin*) = 9.20 µM. The value 112.5% should be disregarded since the maximum value can be 100%

Std Dev standard deviation, *SE* standard error, *CI* confidence interval

except with citric acid (200 mg/L). Unfortunately, we could not induce callus formation after these pre-treatments even though toxic browning was inhibited.

Influence of explant source, culture media, and PGRs

Calli were successfully initiated from embryo and haustoria (Fig. 1). Moreover, such cultures did not produce browning at the conditions tested; hence, the antioxidant treatment was not required. On the other hand, leaves, meristems, and stems did not produce viable callus at the conditions tested irrespectively of the antioxidant pre-treatment. Conversely, Rousset et al. (2003) working with the obligated parasitic weed *Striga hermonthica,* obtained callus from young leaves on MS medium with 2% w/v sucrose, 200 mg/L casein hydrolysate and 0.4% Agargel. Establishment of callus from *Viscum album* was also reported from stems in MS medium plus 5.0 mg/L 2,4-D but at a very low frequency (Lee and Lee 2013).

As for the influence of media and PGRs, the highest frequency of callus induction from embryo was 85% in White media with sucrose 4% w/v as carbon source and NAA:Kin (2.5 µM:9.2 µM) as PGRs (GI = 1.16 ± 0.08) (Fig. 1a, d–f). The result of the full factorial design showed some variability between days (Table 2, column response). Analyzing the ANOVA results (Table 4), the sum of squares for the block effect (day variability) was low compared to other factors. The model was significant ($p < 0.05$ criteria) as well as the factor A (sucrose, $p = 0.015$), the double interaction BC (NAA and Kin, $p = 0.021$) and the triple interaction ABC ($p = 0.041$). A multiple linear equation was built with the information provided by the full factorial experiment allowing us to predict a mean value of callus formation.

$$\begin{aligned} \textit{Callus formation}\ (\%) = {} & -123.3 + 45.9 \times \textit{sucrose} + 18.4 \\ & \times \textit{NAA} + 22.6 \times \textit{Kin} - 5.1 \times \textit{sucrose} \\ & \times \textit{NAA} - 4.8 \times \textit{sucrose} \times \textit{Kin} - 3.1 \\ & \times \textit{NAA} \times \textit{Kin} + 0.7 \times \textit{sucrose} \\ & \times \textit{NAA} \times \textit{Kin} \end{aligned}$$

As an example, using the optimal conditions for this experiment, i.e. sucrose 4% w/v, NAA 2.50 µM, and Kin

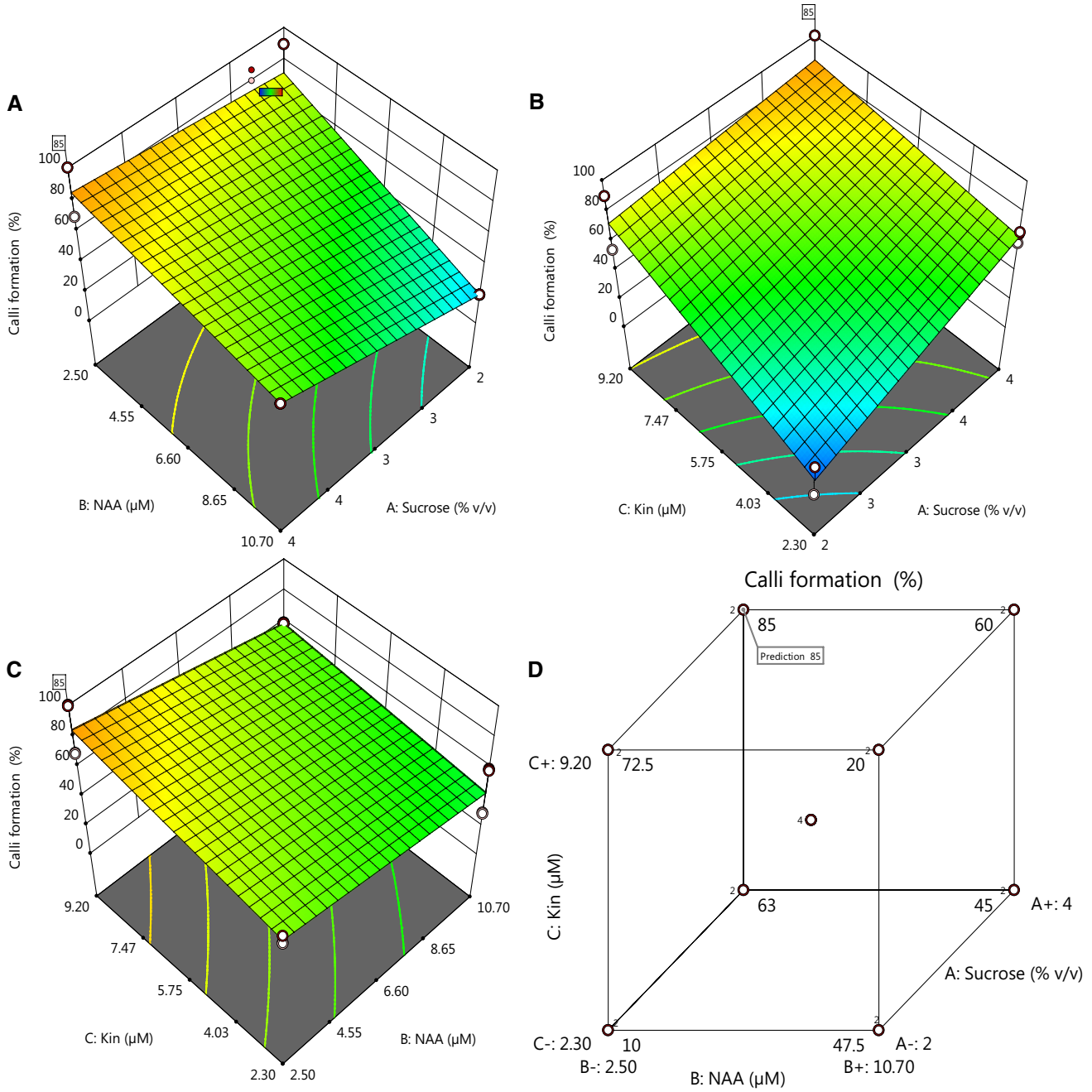

Fig. 2 Graphical representations of the results from the 2^3 full factorial design for initiating *Ligaria cuneifolia* in vitro cultures from embryos. Subfigures **a**–**c** are response surface graphs where the y-axis is the percentage of calli formation and the x-axis and z-axis are the different variables. Subfigure **d** is the tridimensional representation of the design space where each axis represents a variable. y = Kin, x = NAA and A = sucrose. The vertices represent the combination of variables. i.e. top back left vertex corresponds to high level of Kin (9.20 µM), low level of NAA (2.50 µM), and high level of sucrose (4% w/v) giving a response of callus formation of 85%. The number 2 in each vertex represent the number of replicates and the 4 in the center of the cube is the number of center points

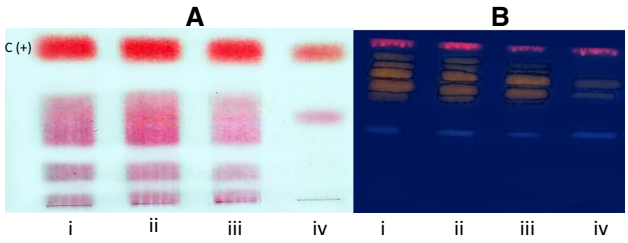

Fig. 3 **a** Monodimensional analysis of proanthocyanidins from *Ligaria cuneifolia* methanolic extracts of leaf (*i*), stem (*ii*), flower (*iii*), and callus (*iv*). The different bands correspond to compounds with different molecular weight (monomers, oligomers and polymers). Catechin [C (+)] was detected in all tissues. Spray reagent: ethanolic solution of vainillin/chlorhydric acid (5% v/v). **b** Monodimensional analysis of flavonols from the methanolic extract of leaf (*i*), stem (*ii*), flower (*iii*), and callus (*iv*). Flavonoids (yellow-orange compounds) and hydroxycinnamic acid derivatives (blue-greenish compounds) are present in all cases. Spray reagent: AEDBE: 2-aminoethyl diphenyl borate ester, Sigma-Aldrich). Wavelength: 366 nm

9.20 µM, we can expect a central value of 85% with a confidence interval (95% CI) between 58 and 100% of callus formation (Table 5). The tridimensional representation of the model is shown in Fig. 2.

The highest frequency of callus formation from haustoria was 35% in Gamborg medium (GI 1.04 ± 0.07) with the addition of sucrose 3% w/v as carbon source and 2,4-D + zeatin (0.45:0.47 µM) as PGRs (Fig. 1a–c). The p values for both tests resulted significant for $p < 0.05$, chi square test $p = 0.00003325$ and Fisher's Exact Test $p = 0.0000007039$ (Table 3).

Our results differ from those obtained with other parasitic or hemiparasitic species. In the case of the obligate parasitic *Orobanche ramosa*, calluses were obtained on TB medium with 0.5 and 1 mg/mL IAA, 20 and 25 mg/L GA3, or 5–15 mg/L Kin (Batchbaroiva et al. 1999). The obligate parasite *Cuscuta reflexa* produced callus from explants of the basal portion of seedlings as well as from shoots (vines) in a modified MS (MMS) medium supplemented with 1 mg/L benzyl adenine (BA) and 3 mg/L NAA (Srivastava and Dwivedi 2001). The Australian mistletoes *Amyema* and *Amylotheca*, developed callus and seedlings on modified MS medium with IAA or NAA (Hall et al. 1987). *Dendrophthoe*, a stem hemiparasitic species produced in vitro cultures in White's medium (Ram and Singh 1991). *Nuytsia*, the largest of all mistletoes produced in vitro cultures on White's medium with casein hydrolysate, IBA, and Kin (Nag and Johri 1976). On the other hand, callus from *Viscum album* L. var. *lutescens* M. was induced on a modified half strength MS medium supplemented with 1.0 ppm Kin and 10.0 ppm NAA (Fukui et al. 1990). In *V. album* L. var. *coloratum*, callus induction was observed in MS medium containing 5.0 mg/L of 2,4-D, but at a very low frequency (4.8%)(Lee and Lee 2013). Other authors (Kim et al. 2008) referred that the frequency of callus formation was higher (27.3%) when *V. album* L. var. *coloratum* flower buds were cultured on B5 medium containing 0.1 mg/L of IAA.

Qualitative and quantitative analysis of polyphenolic compounds

A preliminary screening of polyphenolic compounds by TLC showed the presence of flavonols, hydroxycinnamic acids, proanthocyanidins of high degree of polymerization, and catechin in callus and plant extracts (Fig. 3a, b). The intensity of the bands corresponding to flavonoids and proanthocyanins from callus was lower than those from the plant, and it was higher in the case of hydroxycinnamic acid.

We also performed HPLC MS/MS on the callus and parent plant material for metabolite identification by means of retention time, molecular weight and fragmentation pattern (Fig. 4). Following this, quantification was also carried out by comparison with the respective standard. Results are expressed as µg analyte/g dried extract (mean of four replicates ± SEM) (Table 6). Catechin, quercetin-3-O-glucoside, quercetin-3-O-arabinopyranoside, quercetin-3-O-arabinofuranoside, quercetin-3-O-xyloside, quercetin-3-O-rhamnoside and chlorogenic acid could be identified. These findings agree with previous reports on the wild plant (Dobrecky et al. 2017). It is noteworthy that there is approximately a 5-fold-increase in the content of the detected metabolites in the parent plant. This is expected, given that the parent plant has its full metabolic machinery in place.

Further experiments will be conducted in order to establish cell suspension cultures and to analyze their kinetics of growth and polyphenolics profile and content. Also, analysis of the influence of the host and the phenological state of the wild plant on its polyphenolic content will be performed.

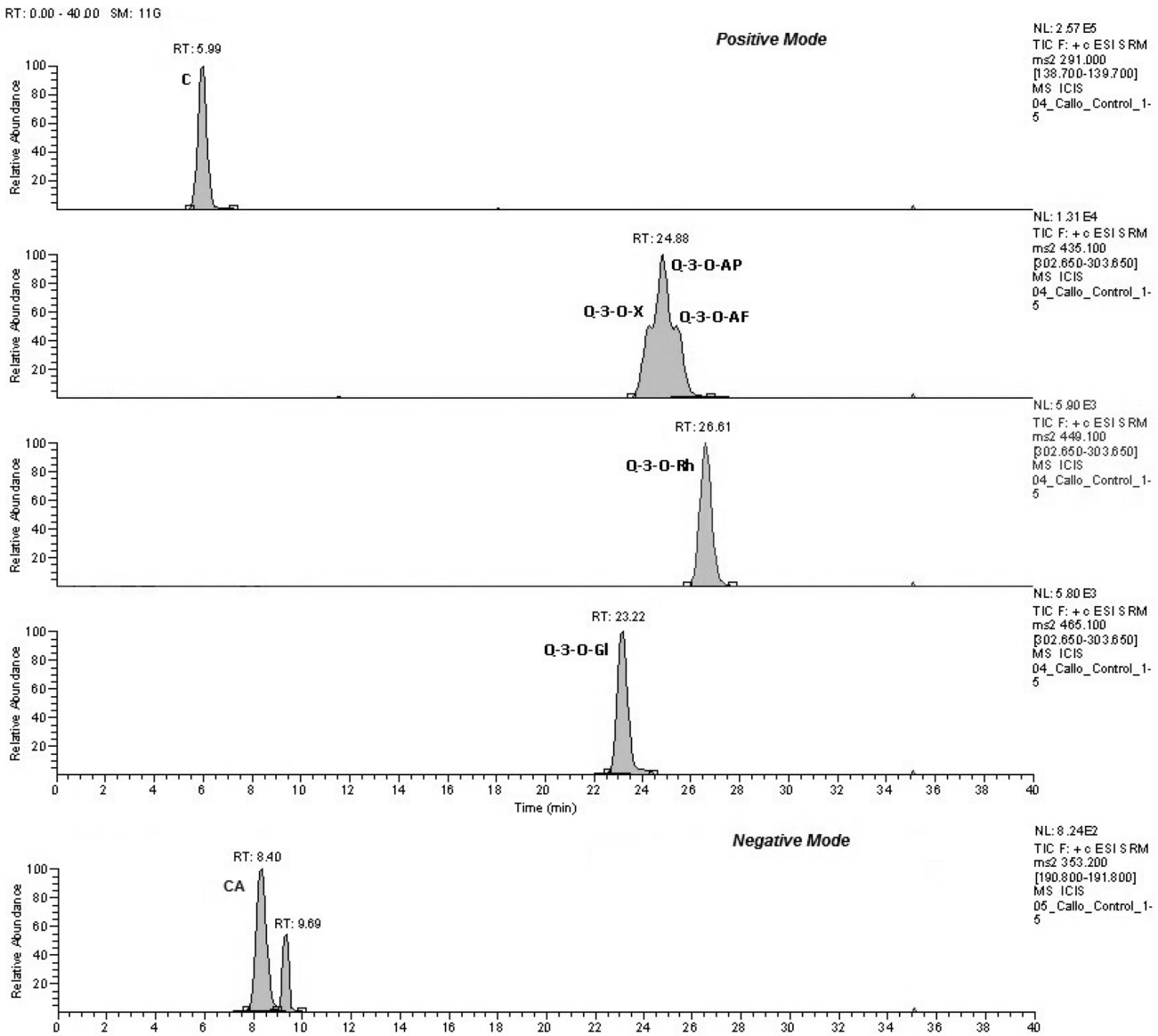

Fig. 4 HPLC–MS/MS chromatogram of the callus extract with identified compounds. Quercetin glycosides were analyzed in positive ionization mode and phenolic acids in negative ionization mode. Catechin (C), quercetin-3-O-glucoside (Q-3-O-Gl), quercetin-3-O-arabinopyranoside (Q-3-O-AP), quercetin-3-O-arabinofuranoside (Q-3-O-AF), quercetin-3-O-xyloside (Q-3-O-X), quercetin-3-O-rhamnoside (Q-3-O-Rh), chlorogenic acid (CA)

Conclusion

The initiation and establishment of *L. cuneifolia* in vitro cultures was challenging. Leaves, stems, and meristems resulted recalcitrant to in vitro culture in the conditions tested. On the other hand, embryos and haustoria succeeded in producing callus. Embryos gave the highest frequency of callus induction (85%) in White medium with sucrose 4% w/v and NAA:Kin (2.5:9.2 µM) as PGRs. In the case of haustoria, the highest frequency of callus induction (35%) was obtained in Gamborg media with sucrose 3% w/v, and 2,4-D and zeatin (0.45:0.47 µM) as PGRs. The HPLC MS/MS analysis unequivocally confirmed the presence of polyphenolic compounds establishing the presence of quercetin glycosides and phenolic acids in callus as was previously seen in the wild plant.

Table 6 Comparative polyphenolic content of calluses and parental plant from *Ligaria cuneifolia*

Analyte	Parent plant (μg/g dried extract)	Callus (μg/g dried extract)
Catechin	2225.7 ± 5.6	464.3 ± 2.2
Q-3-O-glucoside	12.1 ± 4.3	2.2 ± 0.1
Q-3-O-xyloside	28.3 ± 3.7	5.4 ± 0.5
Q-3-O-arabinopyranoside	24.8 ± 4.2	5.2 ± 0.4
Q-3-O-arabinofuranoside	27.6 ± 4.5	5.3 ± 0.5
Q-3-O-rhamnoside	13.4 ± 3.1	2.4 ± 0.3
Chlorogenic acid	31.5 ± 2.9	6.1 ± 0.7

Each value is the mean (4 replicates) ± SEM. quercetin-3-O-glucoside(Q-3-O-glucoside), quercetin-3-O-xyloside(Q-3-O-Xyloside), quercetin-3-O-arabinopyranoside(Q-3-O-arabinopyranoside), quercetin-3-O-arabinofuranoside (Q-3-O-arabinofuranoside), quercetin-3-O-rhamnoside (Q-3-O-rhamnoside)

Results are expressed as mean ± SEM

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Author's contributions MVR and MLB carried out the experiments and participated in drafting the manuscript; CC and FB carried out experiments; ML-B and CD participated in the analysis of the results; AP participated in selecting, collecting, and classifying the plant material; LUS performed statistical analysis; MLW, RAR and MAA initiated the project and supervised the work throughout, MAA also drafted the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

Abiatti D (1946) Las Lorantáceas Argentinas. Rev Mus La Plata NS Sec Bot 7:1–110

Ahmad I, Hussain T, Ashraf I, Nafees M, Maryam RM, Iqbal M (2013) Lethal effects of secondary metabolites on plant tissue culture. Am Eurasian J Agric Environ Sci 13:539–547

Ahmad I, Jaskani MJ, Nafees M, Ashraf I, Qureshi R (2016) Control of Media Browning in Micropropagation of Guava (*Psidium guajava* L.). Pak J Bot 48(2):713–716

Amico GC, Nickrent DL (2007) Phylogeography of the Argentine mistletoe, *Ligaria cuneifolia* (Loranthaceae). Darwiniana 45:55–131

Amuchástegui A, Petryna L, Cantero JJ, Núñez C (2003) Plantas parásitas del centro de Argentina. Acta Botánica Malacitana 28:37–46

Bajaj YPS (1967) In vitro studies on the embryos of two Mistletoes, *Amyema pendula* and *Amyema miquelli*. NZ J Bot 5(1):49–56

Batchbaroiva RB, Slavov SB, Bossolova SN (1999) In vitro culture of *Orobanche ramosa*. Weed Res 39:191–197

Cerdá Zolezzi P, Fernández T, Aulicino PC, Cavaliere V, Greczanik S, Caldas Lopez E, Wagner ML, RIcco RA, Gurni A, Hajos S, Alvarez E (2005) *Ligaria cuneifolia* flavonoid fractions modulate cell growth of normal lymphocytes and tumor cells as well as multidrug resistant cells. Immunobiology 209(10):737–749

Debergh PC, Read PE (1991) Micropropagation. In: Debergh PC, Zimmerman RH (eds) Micropropagation technology and application. Kluwer Academic Publishers, Dordrecht, pp 1–14

Dobrecky CB, Flor SA, López PG, Wagner ML, Lucangioli SE (2017) Development of a novel dual CD-MEKC system for the systematic flavonoid fingerprinting of *Ligaria cuneifolia* (R. et P.) Tiegh-Loranthaceae- extracts. Electrophoresis 38(9–10):1292–1300

Espinosa-Leal CA, Puente-Garza CA, García-Lara S (2018) In vitro plant tissue culture: means for production of biological active compounds. Planta 248:1–8

Fukui M, Azuma JI, Okamura K (1990) Induction of Callus from mistletoe and interaction with its host cells. Bull Kyoto Univ Forests 62:261–269

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res 50:151–158

Gonzálvez J, García G, Galliano S, Dominghini A, Urli L, Monti J, Ronco MT, Frances D, Wagner M, Carnovale C, Luquita A (2017) The enriched proanthocyanidin extract of *Ligaria cuneifolia* shows a marked hypocholesterolemic effect in rats fed with cholesterol-enriched diet. Recent Pat Endocr Metab Immune Drug Discov 11(1):47–53

Hall PJ, Letham DS, Barlow BA (1987) The influence of hormones on development of *Amyema* seedlings cultured in vitro. In: Weber HC, Forstreuter W (eds) Proceedings of the 4th International Symposium on Parasitic Flowering Plants. Philips University, Marburg, pp 285–291

Ishrad M, Rizwan HM, Debnath B, Anwar M, Li M, Liu S, He B, Qiu D (2018) Ascorbic acid controls lethal browning and pluronic F-68 promotes high-frequency multiple shoot regeneration from coltyldonary node explant of okra (*Abelmoschus esculentus* L.). HortScience 53(2):183–190

Johri BM, Bajaj YPS (1962) Behaviour of Mature Embryos of *Dendrophthoe falcata* (l.F) Ettingsh in vitro. Nature 193:194–195

Johri BM, Bajaj YPS (1964) Growth of embryos of *Amyema*, *Anylotheca*, and *Scurrula* on synthetic media. Nature 204:1220–1221

Karunaratne MLWOM, Peries SE, Egodawatta WCP (2014) Callus induction and organogenesis from leaf explants of *Tectona grandis*. Ann Biol Res 5(4):74–82

Khana P, Staba J (1968) Antimicrobials from plant tissue cultures. Lloydia 31:180–189

Kim SW, Ko SM, Liu JR (2008) In vitro seed germination and callus formation on flower bud of Korean mistletoe [*Viscum album* L. var. cololatum (Kom.) Ohwi]. J Plant Biotechnol 35:47–53

Lee KP, Lee DW (2013) The Identification of *in Vitro* Production of Lectin from Callus Cultures of Korean Mistletoe (*Viscum album* L. var. coloratum). Biosci Biotechnol Biochem 77(4):884–887

Majid I, Muhammad J, Rizwan R, Syed ZU, Muhammad SI, Misbah R, Salman M (2014) Effect of plant growth regulators on callus formation in potato. J Agri Food Appl Sci 2:77–81

Martínez GJ (2010) Las plantas en la medicina tradicional de las Sierras de Córdoba. Un recorrido por la cultura campesina de Paravachasca y Calamuchita, Ediciones del Copista, p 212

McHugh ML (2013) The chi square test of independence. Biochemia Medica 23(2):143–149

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15(3):473–497

Nag KK, Johri BM (1976) Experimental morphogenesis of the embryo of *Dendrophtho*e, Taxillus, and Nuytsia. Bot Gaz 37:378–390

Ndakidemi CF, Mneney E, Ndakidemi PA (2014) Effects of ascorbic acid in controlling lethal browning in in vitro culture of *Brachylaena huillensis* using nodal segments. Am J Plant Sci 5(1):5

Nigra HM, Caso O, Giulietti AM (1987) Production of solasodine by calli from different parts of *Solanum eleagnifolium* Cav. plants. Plant Cell Rep 6:135–137

Ohofeghara FA (1971) The effects of growth substances on the growth of *Tapinanthus bauguensis* (Loranthaceae) in vitro. Am Bot 36:563–570

Payne G, Bringi V, Prince C, Shuler M (1991) Quantifying growth and product synthesis: kinetics and stoichiometry. In: Michael S (ed) Plant cell and tissue culture in liquid systems. Hanser/Oxford University Press, Oxford, pp 47–70

R Core Team (2018). R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna. https://www.R-project.org/

RStudio Team (2018). RStudio: Integrated Development for R. RStudio, Inc., Boston http://www.rstudio.com/

Ram RL, Singh MPN (1991) In vitro haustoria regeneration from embryo and in vitro-formed leaf callus cultures in *Dendrophthoe falcata* (L.f.) Ettings. Adv Plant Sci 4:48–53

Rousset A, Simier P, Fer A (2003) Characterization of simple in vitro cultures of *Striga hermonthica* suitable for metabolic studies. Plant Biol 5:265–273

Rustán JJ, Balseiro D, Degrange FJ, Halpern K, Sferco ME, Luna ML, Giudice GE (2003) Características estructurales de los haustorios de *Ligaria cuneifolia* (Loranthaceae) de Argentina. Bol Soc Arg Bot 38:107–108

Scarpa GF, Montani MC (2011) Etnobotánica médica de las "ligas" (Loranthaceae sensu latu) entre indígenas y criollos de Argentina. Dominguezia 27(2):5–19

Sharpe D (2015) Your chi square test is statistically significant: now what? Pract Assess Res Eval 20(8):1–10

Soberón JR, Sgariglia MA, Dip Maderuelo MR, Andina ML, Sampietro DA, Vattuone MA (2014) Antibacterial activities of *Ligaria cuneifolia* and *Jodina rhombifolia* leaf extracts against phytopathogenic and clinical bacteria. J Biosci Bioeng 118(5):599–605

Srivastava S, Dwivedi UN (2001) Plant regeneration from callus of *Cuscuta reflexa*—an angiospermic parasite– and modulation of catalase and peroxidase activity by salicylic acid and naphthalene acetic acid. Plant Physiol Biochem 39:529–538

Stat-Ease, Inc. (2018). Design-Expert Trial (version 11.0.6.0 64-bit)

Trigiano RN (2011) Chapter 12-Propagation of shoot culture. In: Trigiano RN, Gray DJ (eds) Plant tissue culture, development and biotechnology. CRC Press Taylor & Francis Group, Boca Raton, pp 181–192

Varela BG, Fernández T, Taira C, Cerdá Zolezzi P, Ricco RA, Caldas López E, Álvarez E, Gurni AA, Hajos S, Wagner ML (2001) El "muérdago criollo", *Ligaria cuneifolia* (R. et P.) Tiegh -Loranthaceae- Desde el uso popular hacia el estudio de los efectos farmacológico. Dominguezia 17(1):31–50

Vázquez y Novo SP, Wagner ML, Gurni AA, Rondina RVD (1989) Importancia Toxicológica de la presencia de sustancias aminadas en ejemplares de *Ligaria cuneifolia* var. *cuneifolia* colectados en diferentes áreas de la República Argentina. Acta Farmacéutica Bonaerense 8(1):23–29

White PR (1963) The cultivation of animal and plant cells. Ronald Press, New York