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Research Report

In vitro Propagation, Genetic Stability, and Secondary Metabolite Analysis of Wild Lavender (*Lavandula coronopifolia* Poir.)

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Abstract. Lavenders (*Lavandula* species) are important aromatic ornamental medicinal plants with wide ranging applications in perfume and pharmaceutical industries. We developed an in vitro propagation protocol for *Lavandula coronopifolia*. Murashige and Skoog (MS) medium supplemented with 0.5 mg·L^{-1} N^6-benzyladenine was the best medium for the proliferation of microshoots, while the highest rooting frequency was obtained using MS medium supplemented with 1.0 mg·L^{-1} indole-3-butyric acid. Inter-simple sequence repeat analysis revealed that the in vitro-propagated microshoots were highly genetically stable, even after subculture. The highest callus fresh weight (667.9 mg) was obtained by propagating on medium supplemented with a combination of 1.0 mg·L^{-1} naphthaleneacetic acid and 0.5 mg·L^{-1} butyric acid. Using the Folin-Ciocalteu method, methanolic extracts of wild *L. coronopifolia* revealed total phenolic content of 4.9 mg expressed in gallic acid equivalents (GAE) (mg GAE·g^{-1} dry matter). Radical scavenging activity was estimated at 85% using the free radical 2,2-diphyenyl-picrylhydrazyl assay. Using the brine shrimp assay for cytotoxicity, the methanolic extract was found to be nontoxic. Finally, liquid chromatography tandem mass spectrometry with standard reference compounds was used to quantify the key phenolic compounds in both in vitro and in vivo-grown *L. coronopifolia*. Six major phenolic compounds were identified: caffeic acid, rosmarinic acid, rutin, quercetin, luteolin, and hesperidin. Levels of these phenolic compounds were highest in wild plant extracts.

***Additional key words*:** antioxidant, callus, liquid chromatography tandem mass spectrometry, microshoots, total phenolic content

Introduction

Plants have been used in medicine, nutrition, flavorings, and cosmetics for thousands of years. Medicinal plants are rich in bioactive compounds with diverse pharmacological actions, including antibacterial, antifungal, antispasmodic, antioxidant, and antiproliferative activities (Dragland et al., 2003). Medicinal plants are also a rich source of phenolic compounds and essential oils. The antioxidant activities of natural phenolic compounds are attributed to their redox properties, as they are important for neutralizing free radicals, quenching singlet and triplet oxygen, and decomposing peroxides (Zheng and Wang, 2001).

Continuous efforts are made to improve the quality and productivity of phenolic-containing medicinal plants. Techniques including tissue culture of shoots, roots, calli, and cell suspension cultures are used to develop desirable plant traits (Thorpe, 2007). *Lavandula coronopifolia* (Arabic: Khozama) is a perennial herb in the *Lamiaceae* family. Historically, it has been used as a food, as a drug for the treatment of infections, as perfume, and in soap manufacture. It flourishes mainly in the Middle East, and in the North and West of Africa. It grows in deep sandy soils (Singh et al., 2007), and is cultivated around the world in both arid and semi-arid climates. It has a life span of about 10 years, and is resistant to drought and high temperatures (Hassanpouraghdam et al., 2011).

In 2014, *L. coronopifolia* was red-listed as a near-threatened plant in Jordan. The in vitro propagation of *L. coronopifolia* is required to avoid its extinction, but there are few reports in the literature where this has been done.

In this study, we established an in vitro propagation

protocol for *L. coronopifolia* Poir, and compared the phytochemical activities of in vitro-grown microshoots and calli with those of wild-grown samples.

Materials and Methods

Plant Materials

Aerial parts of *L. coronopifolia* were collected from Aqaba in southern Jordan (29°29'0.10"N, 35° 4'46.65"E) during December 2014 (Fig. 1A). *L. coronopifolia* samples were air-dried in the shade, seeds were collected and stored for later in vitro propagation (Kanaan, 2016). Shoot tissues were ground and stored in the refrigerator for later biological activity experiments.

Seed Germination

L. coronopifolia seeds were washed with a sterile solution of 70% ethanol and detergent for 15 min, then washed with 70% alcohol for 5 min, followed by 100% ethanol for a few seconds. Seeds when then immediately spread on to sterilized filter paper in a laminar air-flow cabinet to dry. Sterilized seeds were inoculated on to Petri dishes containing half-strength Murashige and Skoog (MS) medium (Murashige & Skoog, 1962). Sucrose was used as the carbon source (15 $g \cdot L^{-1}$), and the pH of the medium was adjusted to 5.8. Agar (7 $g \cdot L^{-1}$) was added to solidify the medium. The medium was autoclaved at 121°C and 1.15 $kg \cdot cm^{-2}$ pressure for 20 min. Inoculated plates were incubated in a growth chamber at 24 ± 2°C under a 16 h light/8 h dark photoperiod. For comparison by liquid chromatography tandem mass spectrometry (LC-MS/MS), another group of seeds was grown in potting soil in a greenhouse.

In vitro Shoot Culture

Shoot tips (Fig. 1B) of 3-week-old in vitro-grown seedlings were excised and cultured on full-strength MS medium supplemented with different levels of two growth regulators: benzyl adenine (BA) and kinetin (Kin) at 0.0, 0.5, 1.0 and 2.0 $mg \cdot L^{-1}$. The pH of the medium was adjusted to 5.8, and autoclaved as previously described. Cultures were incubated in a growth chamber at 24 ± 2°C and under a 16 h light/8 h dark photoperiod. After culturing for 8 weeks, the length

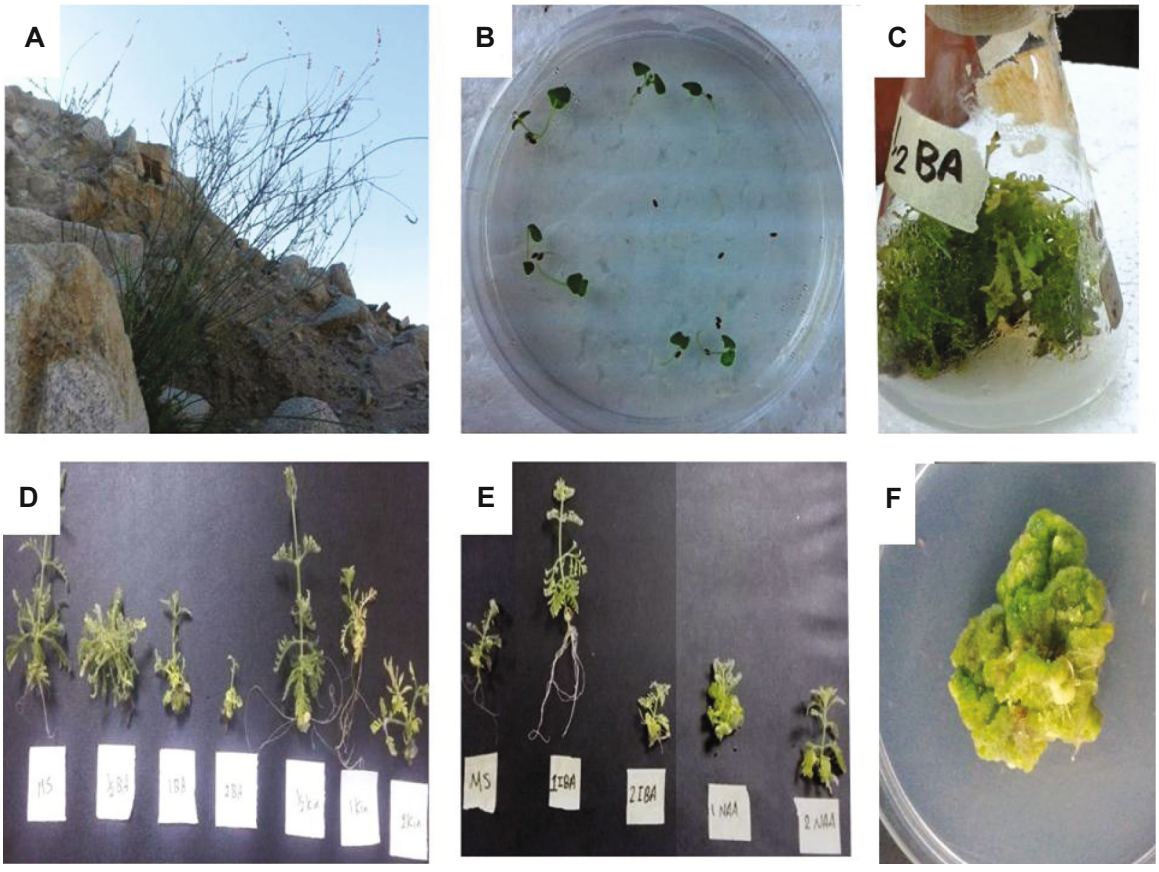

Fig. 1. Wild Lavender and in vitro propagation (A) *Lavandula coronopifolia* Poir, collected from the Aqaba region of Jordan. (B) *L. coronopifolia* seeds cultured on Murashige and Skoog (MS) medium. (C) Microshoots cultured on half-strength N^6-benzyladenine (BA) medium. (D) *L. coronopifolia* shoot proliferation on MS medium supplemented with different concentrations of MS, BA and kinetin (Kin). (E) *L . coronopifolia* root induction on MS medium supplemented with different concentrations of indole-3-butyric acid (IBA) and naphthaleneacetic acid (NAA). (F) *L. coronopifolia* calli grown on MS medium supplemented with a combination of 1.0 $mg \cdot L^{-1}$ NAA + 0.5 $mg \cdot L^{-1}$ BA.

and number of microshoots and number of leaves were measured and recorded.

In vitro Root Culture

Shoot tips of 3-week-old in vitro-grown seedlings were excised and cultured on full-strength MS medium supplemented with different levels of 1-naphthaleneacetic acid (NAA) or indole-3-butyric acid (IBA) at 0.0, 0.5, 1.0 and, 2.0 $mg \cdot L^{-1}$. The pH of the medium was adjusted to 5.8 and autoclaved as described previously. Cultures were incubated in a growth chamber at 24 ± 2°C and under a 16 h light/8 h dark photoperiod. After culturing for 8 weeks, the number and length of roots and number of microshoots were measured and recorded.

Callus Induction

Leaves were excised from in vitro-grown *L. coronopifolia* plants and inoculated on full-strength MS medium supplemented with different levels of 2 4-dinitrophenylhydrazine (2,4-D; 0, 1, 2 and 3 $mg \cdot L^{-1}$), and different combinations of BA and NAA (0.5 $mg \cdot L^{-1}$ BA + 0.5 $mg \cdot L^{-1}$ NAA, 1 $mg \cdot L^{-1}$ BA + 1 $mg \cdot L^{-1}$ NAA, 1 $mg \cdot L^{-1}$ BA + 0.5 $mg \cdot L^{-1}$ NAA, and 0.5 $mg \cdot L^{-1}$ BA + 1 $mg \cdot L^{-1}$ NAA). The pH of the medium was adjusted to 5.8. Cultures were incubated in a growth chamber at 24 ± 2°C under a 16 h light/8 h dark photoperiod, or in complete darkness. After 7 weeks, the callus fresh weight, texture and induction rate were measured and recorded.

Callus Growth Kinetics

A callus growth curve was established by growing several callus subcultures on MS medium supplemented with 1.0 $mg \cdot L^{-1}$ NAA + 0.5 $mg \cdot L^{-1}$ BA. Three randomly chosen calli were incubated in a growth chamber at 24 ± 2°C under light. Fresh weights of calli were measured and recorded every 7 d for 63 d. Callus growth rate data were recorded weekly.

Plant Material and Methanolic Extraction

Wild plants, in vitro-grown microshoots, and calli were dried in an oven at 35°C for 24 h, then ground for further analysis. About 10 g of fine powder of each of wild plant material, in vitro microshoots and calli were extracted with 100 mL methanol by continuously shaking at room temperature for 24 h. A rotary evaporator was used to remove the solvent under pressure at 45°C. The resulting dry extracts were stored at -18°C until required for analysis.

Antioxidant Activity

The antioxidant activities of *L. coronopifolia* extracts were measured using the free radical 2,2-diphyenyl-picrylhydrazyl (DPPH) method, as described by Almey et al. (2010) About 6 mg of DPPH was dissolved in 100 mL methanol. About 1 mL (0.1 $mg \cdot L^{-1}$) from each methanolic extract was mixed with 2 mL of DPPH solution. The absorbance of each mixture was measured using a Genesys 10S UV-Vis spectrophotometer at 517 nm after 30 min of incubation at room temperature. Ascorbic acid was used as a control.

The radical scavenging activity of each extract was calculated using the equation:

Scavenging activity (%) = (1 - Absorbance of sample/Absorbance of control) × 100%

Total Phenolic Content

Total phenolic content was calculated using the Folin-Ciocalteu method (Stanković, 2011). Briefly, about 100 µL of each methanolic extract (1 $mg \cdot L^{-1}$) was mixed with 0.75 ml 10% Folin-Ciocalteu's (FC) reagent. After 5 min, 0.75 mL of 6% sodium carbonate (Na_2CO_3) solution was added. After incubating for 90 min, the absorbance of the blue mixture was measured using a Genesys 10S UV-Vis spectrophotometer at 765 nm. Total phenolic content was expressed in terms of gallic acid equivalents (GAE) (mg GAE $\cdot g^{-1}$ dry matter) using a reference curve of different concentrations of gallic acid.

Brine Shrimp Lethality Test

Brine shrimp (*Artemia salina*) eggs were purchased from Ocean Star International, Inc. (USA) and stored at 4°C. Eggs were hatched in a shallow rectangular container with a plastic divider and multiple holes, containing 38% artificial sea water, Nauplii hatching time was 48 h at room temperature. A stock solution of 80 mg of each plant extract was dissolved in 2 mL dichloromethane (CH_2Cl_2) and used for serial dilution to make concentration of 10, 100, and 1000 $\mu g \cdot mL^{-1}$. The CH_2Cl_2 was then allowed to evaporate, and ten hatched larvae were added to each extract concentration. The final volume was 5 mL of artificial sea water. Colchicine was used as a positive control and sea water as a negative control. The number of dead (immobile) larvae was counted after 24 h (Tawaha et al., 2006). The lethal concentration (LC_{50}) of each plant extract was calculated in $mg \cdot mL^{-1}$.

Phytotoxic Activity

Radish (*Raphanus sativus* L. var. national) seeds were sterilized using 70% ethanol for 1 min, then washed three times with sterile distilled water. Methanolic extracts of wild plants, microshoots and calli at different concentrations (0.2, 0.4, 0.6 $mg \cdot mL^{-1}$ dissolved in distilled water) were added to seeds in Petri dishes, then incubated in a completely dark growth chamber at 25°C. Percentage germination and root length was measured and calculated after 7 d. Distilled water was used as a control (Julio et al., 2015).

Liquid Chromatography-mass Spectrometry (LC-MS) Analysis

Using negative and positive ionization modes, LC-MS was used to qualitatively and quantitatively analyze phenolic compounds in methanolic extracts of *L. coronopifolia* collected from wild habitats, and calli, microshoots, and greenhouse-grown plants. Major compounds in crude extracts were identified by comparing retention times, molecular weights (MT) and in-source MS fragments, with those of reference standard compounds. Identified compounds were then quantified using calibration curves of authentic reference standards. Electrospray ionization in negative ion multiple reaction monitoring mode was utilized for mass detection.

About 0.5 g methanolic extract from in vivo and in vitro-grown plants was dissolved in 3.0 mL methanol, and filtered through 0.45-μm pore size filters. The filtrate was then injected into an LC-MS system (Agilent 1200 series LC-MS/MS triple quadrupole API 3200) connected to a binary pump (G1312A) for high-pressure solvent delivery. A Theromo Hypersil (ODS) column (150 × 4 mm, 5 μm) was used. The mobile phase consisted of (A) 90:10 distilled water and formic acid, and (B) 90% methanol, 0.1% formic acid and CH_3CN. Stock solutions of reference standards used were: quercetin, P-coumaric acid, gallic acid, rosmarinic acid, luteolin, rutin, caffeic acid, sinapic acid, apigenin, vanillic acid, epicatechin, chlorogenic acid, diosmaric acid, ferulic acid, hesperidin, and naringenin. The concentration of each phenolic compound in each sample was calculated using the equation:

$$\% W/W = C \times FV \times 100\% / W$$ (Alali et al., 2006)

where C is the phenolic concentration of the sample (mg·mL^{-1}), extrapolated from the calibration curve's linear regression; FV is the final volume of the sample in mL; and W is the sample weight in mg.

Analysis by Inter-simple Sequence Repeat Polymerase Chain Reaction (ISSR-PCR)

L. coronopifolia microshoots grown in vitro and subcultured more than 5 times (each subculture grown for 4 weeks) and mother plant material were used in this experiment. Fresh leaves from each plant were used for DNA extraction. Leaves (0.5 g) were ground with 600 μL of cetyl trimethylammonium bromide (CTAB) extraction buffer and incubated in a water bath at 55°C for 15 min, before adding 600 μL of $CHCl_3$ and centrifuging for 2 min at 1400 rpm to recover the supernatant. Fifty microliters of sodium acetate and 500 μL of isopropanol were added, and this was centrifuged at 1400 rpm for 1 min to isolate the pellet, then 500 μL of 70% ethanol was added before centrifuging again at 1400 rpm for 1 min. Finally, 50 μL of nuclease-free water was added. Samples were stored at -20°C. The purity and concentration of extracted DNA was evaluated by spectrophotometry and gel electrophoresis.

DNA amplification was performed in a thermal cycler (Gene Pro, model TC-E-96G). Twenty-two primers were used for DNA amplification. The reaction contents were: 12.5 μL of a mixture of Taq DNA polymerase, $MgCl_2$, buffers and deoxyribonucleotide triphosphate; 1.5 μL primer, and 2 μL DNA, and this was made up to 25 μL with nuclease-free water. Thermal cycling was programmed at: 94°C for 5 min, followed by 35 denaturation cycles at 49°C for 40 s, 1 min at annealing temperature (specific for each primer; see Table 1), and extension at 72°C for 1 min. Finally, the reaction mixture was incubated at 72°C for 5 min and

Table 1. Primers used in ISSR amplification

Primer	Sequence (5' → 3')	Annealing temp. (°C)	No. of monomorphic bands	No. of polymorphic bands	Total no. of amplified bands
UBC807	(AG)7T	51	7	0	7
UBC810	(GA)8T	51	9	2	11
UBC826	(AC)8C	54	8	0	8
UBC828	(TG)8A	52	6	0	6
UBC834	AG)8Y**T8	52	10	4	14
UBC843	(CT)8RA	52	9	0	9
UBC845	(CT)8RG	52	10	0	10
UBC847	(CA)8	51	7	0	7
UBC848	(CA)8R*G	52	7	0	7
UBC857	(ACA)5CYG	52	3	0	3
UBC861	(ACC)6	55	8	0	8
UBC864	(ATG)6	55	8	3	11
UBC880	(GGA)5	52	5	0	5
UBC881	(GGGGT)3	52	9	0	9
UBC890	(GT)7	56	8	0	8
Total no. of bands produced			114	9	123

R*= (A, G)
Y**= (C, T)

stored at 4°C.

Stained (1.5%) agarose gel electrophoresis at 100 V was used, with tris-borate-ethylenediaminetetraacetic acid (1X) as running buffer. Gel products were visualized under UV light and analyzed for the presence or absence of bands for mother and in vitro-propagated plants.

Statistical Analysis

All experiments were performed in triplicate using a completely randomized design. Each treatment was replicated at least five times. Means, standard deviations, standard errors and analysis of variance (ANOVA) were calculated using SPSS software version 16.0 (2007). Means were separated using Tukey's range test at $p < 0.05$.

Results

Shoot Proliferation

The highest number of microshoots (3.6 microshoots per explant) were seen on MS medium supplemented with 0.5 mg·L^{-1} BA , compared to only 1 microshoot on control medium (Figs. 1C, 1D, 2A). No significant differences were observed between the number of proliferated microshoots grown on control medium and those grown on medium supplemented with 1.0 or 2.0 mg·L^{-1} BA or Kin. Adding BA or Kin to growth medium significantly inhibited shoot elongation. Microshoots grown on MS medium supplemented with Kin were longer than those grown on MS medium supplemented with BA (Fig. 2B).

There were no significant differences in the number of leaves grown on control medium or 0.5 mg·L^{-1} BA (about 17 leaves per explant). Higher levels of Kin or BA significantly inhibited leaf formation (Fig. 2C).

Root Formation

MS medium supplemented with IBA induced root formation in *L. coronopifolia*. The maximum number of roots was observed at 1.0 mg·L^{-1} IBA. Adding NAA to MS medium resulted in complete inhibition of root formation (Figs. 1E, 3A). The length of *L. coronopifolia* roots grown on MS medium supplemented with IBA was longer than those grown on control medium and medium supplemented with high levels of NAA (1.0 and 2.0 mg·L^{-1}). The longest root observed (5.7 cm) was grown on 1.0 mg·L^{-1} IBA medium, followed by 2 cm on 2.0 mg·L^{-1} IBA medium, while MS medium supplemented with NAA only induced root formation at a concentration of 0.5 mg·L^{-1} (Fig. 3B).

Callus Induction

Leaves and stems from in vitro-grown *L. coronopifolia* microshoots were used to assess callus induction. In general, leaves were better at forming calli than stem explants under all combinations of growth regulators (Table 2). Callus induction rates for both stem and leaf explants were also better under light conditions than dark conditions. All combinations of growth regulators used induced the growth of soft, healthy, green calli (Fig. 1F). The control medium resulted in the least number of calli compared to other medium combinations. MS medium supplemented with 1.0 mg·L^{-1} NAA + 0.5 mg·L^{-1} BA was the best combination, inducing the formation of large, green, highly friable calli with the maximum recorded fresh weight (667.9 mg) and the largest leaf surface area under light conditions. This was followed by the combination of 1 mg·L^{-1} NAA + 1 mg·L^{-1} BA (Table 2).

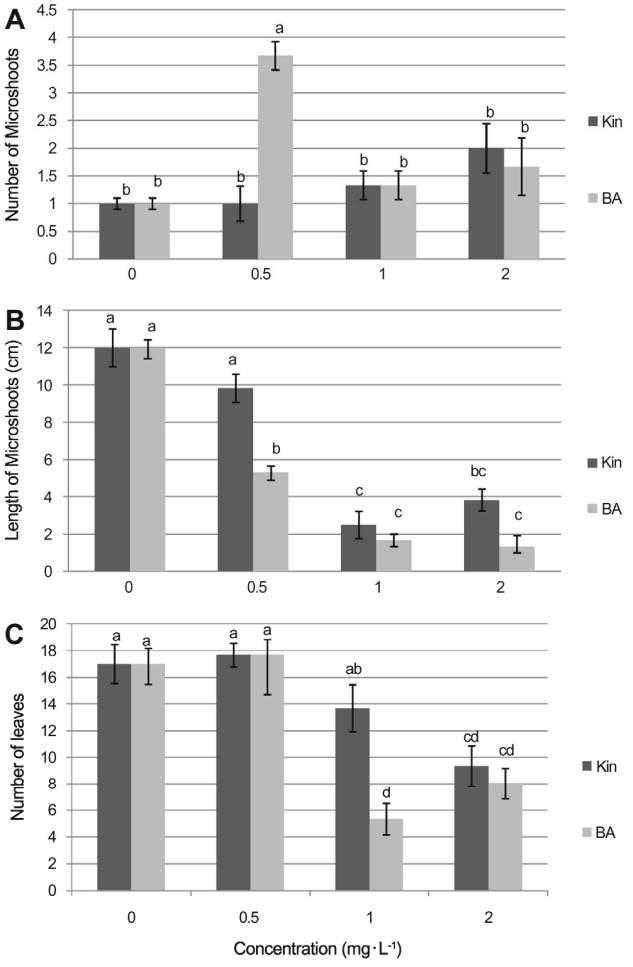

Fig. 2. Proliferation of *L. coronopifolia* shoots grown on media supplemented with different levels (0, 0.5, 1.0, 2.0 mg·L^{-1}) of N^6-benzyladenine (BA) and kinetin (Kin). (A) Number of *L. coronopifolia* microshoots grown on MS medium supplemented with different levels of BA and Kin. (B) Length of *L. coronopifolia* microshoots grown on MS medium supplemented with different levels of BA and Kin. (C) Number of leaves of *L. coronopifolia* microshoots grown on MS medium supplemented with different levels of BA and Kin. Error bars represent standard error (n = 3). Means with different lower case letters are considered significantly different according to Tukey's range test at $p < 0.05$.

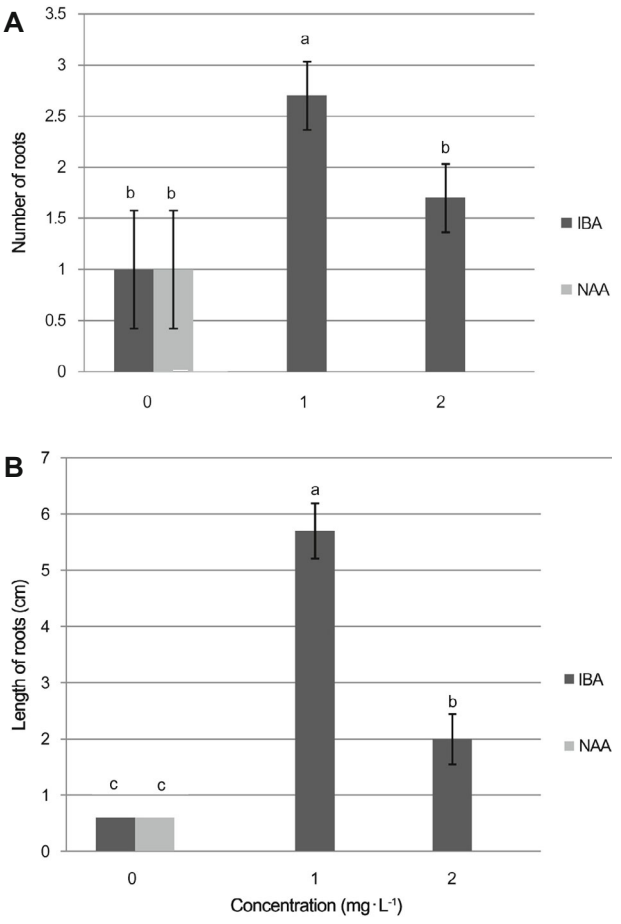

Fig. 3. Proliferation of *L. coronopifolia* roots grown on media supplemented with different levels (0, 1.0, 2.0 mg·L^{-1}) of indole-3-butyric acid (IBA) and naphthaleneacetic acid (NAA). (A) Number of *L. coronopifolia* roots grown on Murashige and Skoog (MS) medium supplemented with different levels of IBA and NAA. (B) Length of *L. coronopifolia* roots grown on MS medium supplemented with different levels of IBA and NAA. Error bars represent standard error (n = 3). Means with different lower case letters are considered significantly different according to Tukey's range test at $p < 0.05$.

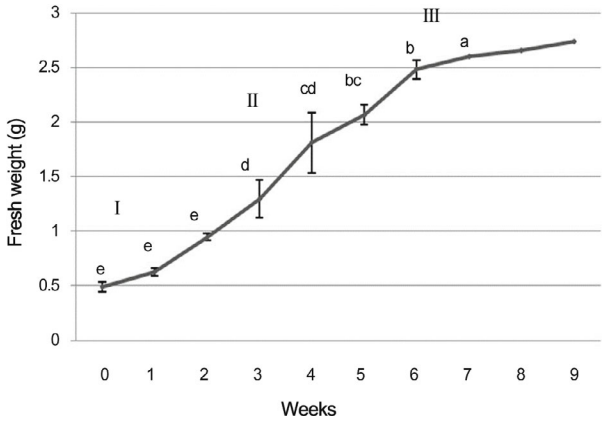

Fig. 4. Growth kinetics of *L. coronopifolia* calli grown on Murashige and Skoog (MS) medium supplemented with 1.0 mg·L^{-1} NAA + 0.5 mg·L^{-1} BA. I, lag phase; II, exponential phase; III, stationary phase. Error bars represent standard error. Means with different lower case letters are considered significantly different according to Tukey's range test at $p < 0.05$.

Callus Growth Kinetics

L. coronopifolia calli grown on MS medium supplemented with 1.0 mg·L^{-1} NAA + 0.5 mg·L^{-1} BA were used to establish a callus growth curve. The growth curve showed an S-shaped pattern; the lag phase (I), exponential phase (II) and stationary phases (III) can be seen in Fig. 4. The best growth rate was observed between weeks 2 and 4.

Genetic Stability of in vitro-propagated Plants

The integrity of *L. coronopifolia* DNA was examined after each of several 4-week subculture passages, ISSR was used as a molecular marker. Gel electrophoresis of all the tested primers showed successful amplification in either mother plant material or regenerated microshoots. Of the 22 primers used for ISSR analysis, only 15 produced reproducible and scorable bands. In total, 123 amplification bands were produced

Table 2. Callus fresh weight (mg) of *L. coronopifolia* grown under light or dark conditions using MS medium supplemented with different levels and combinations of 2.4-D, NAA and BA

Media	Callus Fresh Weight (mg)			
	Dark		Light	
	Stem	Leaf	Stem	Leaf
MS	34.4	16.3	23.7	42.8
1 mg·L^{-1} 2,4-D	130.8	188.1	187.4	322.3
2 mg·L^{-1} 2,4-D	104.1	221.8	149.5	267.4
3 mg·L^{-1} 2,4-D	217.6	197.8	175.6	377.9
0.5 mg·L^{-1} NAA + 0.5 mg·L^{-1} BA	91.9	84.6	466.3	333.3
1 mg·L^{-1} NAA + 0.5 mg·L^{-1} BA	96.3	104.9	270.7	667.9
1 mg·L^{-1} NAA +1 mg·L^{-1} BA	71.9	77.9	417.9	546.4
0.5 mg·L^{-1} NAA + 1 mg·L^{-1} BA	74.3	120.3	428.6	525.4

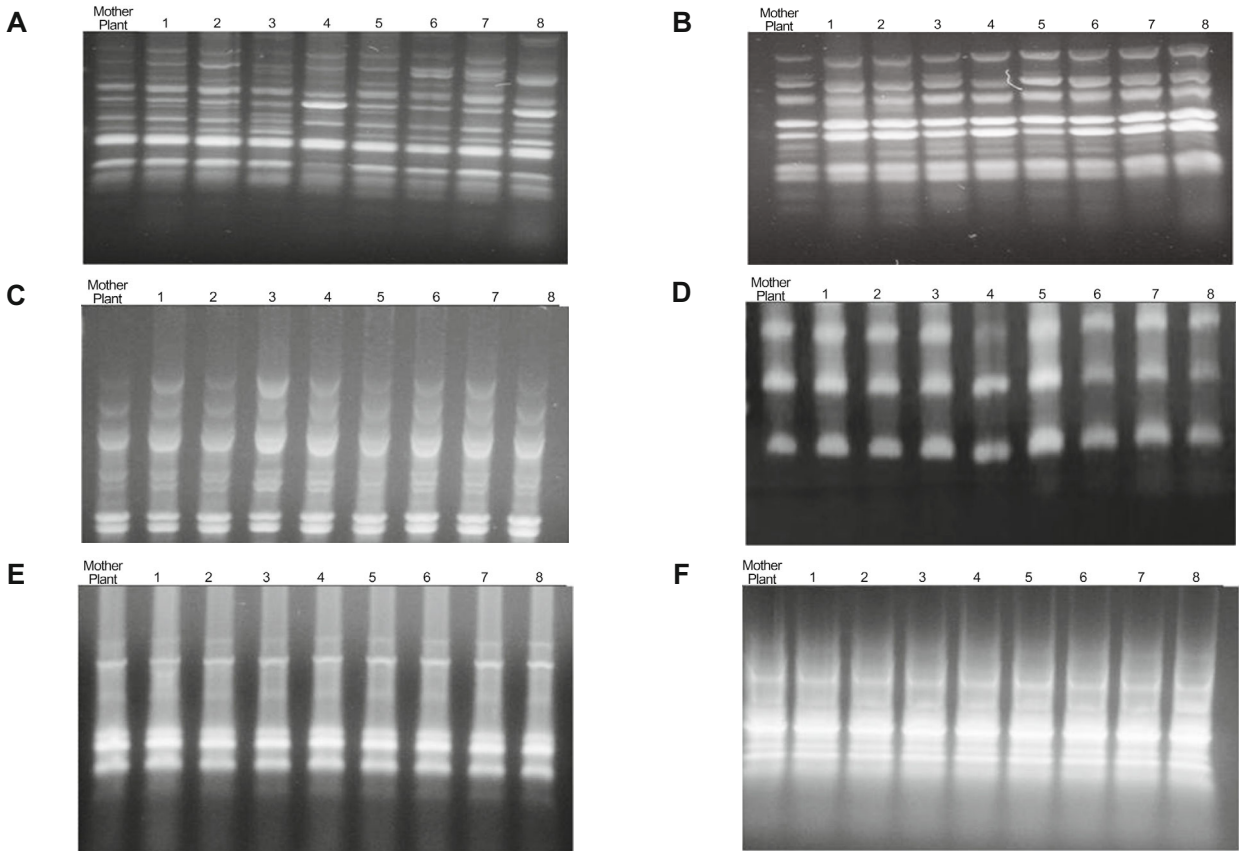

Fig. 5. Banding patterns of *L. coronopifolia* primers after a series of subcultures, determined by inter-simple sequence repeat polymerase chain reaction (ISSR-PCR). Primers (A) UBC834; (B) UBC810; (C) UBC881; (D) UBC857; (E) UBC807; and (F) UBC861.

from these 15 primers, with the number of bands per primer ranging from 3 to 14. Nine bands were polymorphic and the remaining 114 were monomorphic (Table 1). Primers UBC 807, UBC 826, UBC 828, UBC 843, UBC 845, UBC 847, UBC 848, UBC 857, UBC 861, UBC 880, UBC 881, and UBC 890 were entirely monomorphic for both the mother plant and in vitro-propagated plants (Figs. 5C-F). In contrast, primers UBC 810, UBC 834 and UBC 864 exhibited polymorphisms (Figs. 5A, 5B). Banding patterns were highly uniform between mother plant and in vitro-propagated microshoots, indicating a high degree of genetic fidelity when *L. coronopifolia* was micropropagated for several passages.

Antioxidant Activity and Total Phenol Content

The antioxidant activity of *L. coronopifolia* against reactive oxygen species (ROS) was examined using the DPPH radical-scavenging activity method. Methanolic extracts of wild plants, microshoots, and calli were used. The highest rate of radical-scavenging activity ($p < 0.05$) was found in wild plant extract (85%), followed by calli (30%) and microshoots (23%) (Fig. 6A).

The total phenol content of *L. coronopifolia* was determined using the Folin-Ciocalteu method. The highest levels ($p < 0.05$)

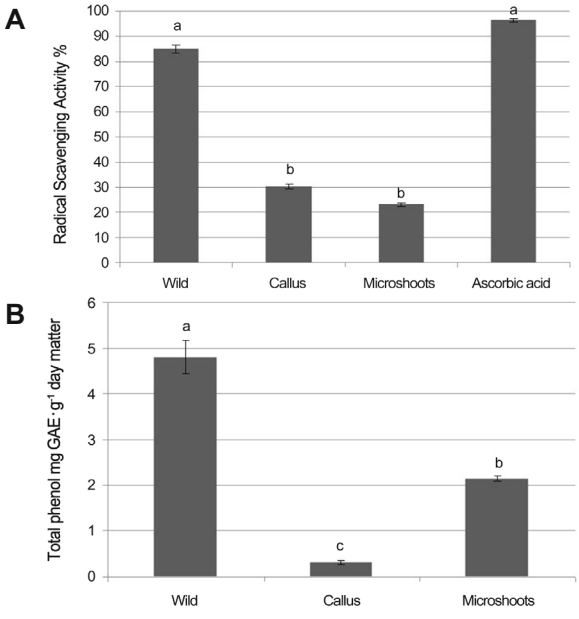

Fig. 6. Phytochemical activities of *L. coronopifolia* extracts. (A) Antioxidant activity (radical-scavenging activity %) of different *L. coronopifolia* extracts measured using the 2,2-diphyenyl-picrylhydrazyl (DPPH) method. (B) Total phenol content (mg GAE·g^{-1}) of different *L. coronopifolia* extracts measured using Folin-Ciocalteu reagent. Error bars represent standard error (n = 3). Means with different lower case letters are considered significantly different according to Tukey's range test at $p < 0.05$.

Table 3. Brine shrimp lethality test results

Sample	LC_{50} (μg·mL^{-1})	Average number of survivals after 24 h		
		1000 μg·mL^{-1}	100 μg·mL^{-1}	10 μg·mL^{-1}
Wild plant	>1000	8.3	9.3	9.7
Callus	>1000	7	8.7	9
Microshoots	>1000	7.7	9	9
Colchicine		0		
Sea water		10		

Table 4. Phytotoxic effect of extracts of *L. coronopifolia* wild plant, microshoot and callus on the germination of radish seeds

Concentrations (mg·mL^{-1})	Germination %			Radical root length (cm)		
	Microshoots	Wild plant	Callus	Microshoots	Wild plant	Callus
0	90 a*	90 a	90 a	6.9 a	6.9 a	6.9 a
0.2	80 a	70 b	90 a	4.3 b	3.7 b	4.8 b
0.4	80 a	70 b	90 a	2.9 c	2.1 c	3.5 c
0.6	80 a	70 b	90 a	2.9 c	1.1 d	3.5 c

* Means followed by identical letters are not considered significantly different according to Tukey's range test at $p < 0.05$.

of phenolic compounds were observed in wild plants (4.9 mg GAE·g^{-1}), followed by microshoots (2.1 mg GAE·g^{-1}), and the lowest was observed in calli (0.4 mg GAE·g^{-1}) (Fig. 6B).

Brine Shrimp Lethality Test

Brine shrimp cytotoxicity bioassays were used to assess the cytotoxicities of methanolic extracts of wild plants, microshoots, and calli. All three tested extracts were nontoxic with LC_{50} values above 1000 μg·mL^{-1} (Table 3).

Biological Phytotoxic Activity

The phytotoxic effect of *L. coronopifolia* was tested against radish (*Raphanus sativus*) seeds. Only wild-grown *L. coronopifolia* had a significantly toxic effect on radish germination (Table 4). Extracts from in vitro-grown plants were less toxic than wild-grown plant extracts. At 0.6 mg·L^{-1} of wild plant extract, 70% of seeds germinated and the length of radical roots was 1.1 cm; when distilled water was used as a control, 90% of seeds germinated and the radical root length was 6.9 cm. Callus extract resulted in root length of 3.5 cm with 90% germination (Table 4).

Liquid Chromatography-mass Spectrometry (LC-MS)

LC-MS was used to qualitatively analyze extracts of in vitro-grown microshoots and wild, in vivo-grown *L. coronopifolia*. Comparison of total ion chromatograms (TICs) profiles (fingerprints), in terms of chromatographic peak retention times and the corresponding molecular ion peaks, revealed high similarity with differences in peak areas. In general, peak areas were more intense in in vitro microshoots compared with wild plants (Fig. 7).

Using LC-MS/MS to compare retention times and molecular weights of selected phenolic compounds in extracts of wild plants, calli, microshoots and greenhouse-grown *L. coronopifolia* with corresponding reference standards. six major phenolic compounds were revealed: caffeic acid, rosmarinic acid, rutin, quercetin, luteolin, and hesperidin (Fig. 7 and 8). Estimating the concentrations of these identified compounds using calibration curves of corresponding reference compounds showed that luteolin was the most abundant phenolic compound in wild plants, with a concentration of 370 μg·g^{-1} dry extract (Table 5). Hesperidin was the least abundant, with a concentration of 0.5 μg·g^{-1} dry extract in greenhouse-grown plants. In general, levels of rutin, quercetin, luteolin, and hesperidin were higher in wild plant samples, while caffeic acid and rosmarinic acid were higher in in vitro-grown plant samples (Fig. 8, Table 5).

Discussion

In vitro Propagation of *L. coronopifolia*

There have been few studies on the in vitro propagation of *L. coronopifolia*. In this study, we established a simple and efficient *L. coronopifolia* micropropagation protocol. Plant tissue culture techniques are useful for the mass-scale propagation of plants within a shorter timeframe than traditional methods. In vitro propagation can also reduce diseases in propagated plants.

In our study, *L. coronopifolia* seeds were germinated on half-strength MS medium; Pandey et al. (2013) found this medium to be the best for germinating *Psoralea corylifolia*

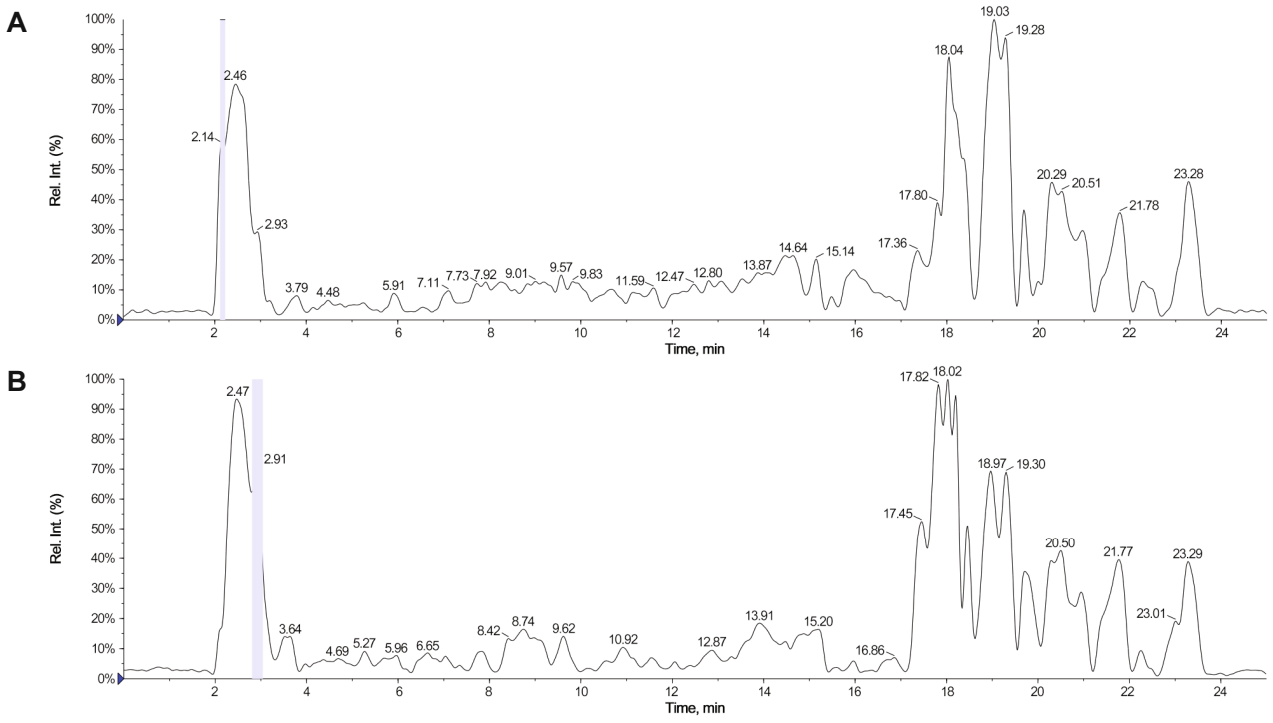

Fig. 7. (-)-ESI total ion chromatograms. (A) Crude extract of wild *L. coronopifolia*; (B) crude extract of *L. coronopifolia* microshoots.

Table 5. Concentrations of phenolic compounds in *L. coronopifolia* extracts grown under different conditions, determined by LC-MS

Sample	Concentration μg·g^{-1} dry extract weight					
	Hesperidin	Luteolin	Quercetin	Rutin	Rosmarinic acid	Caffeic acid
Wild plant	21 a*	370 a	1.4 a	29 a	13 c	17 c
Greenhouse	0.2 d	78 c	0.5 b	0.5 c	1 d	11 d
Callus	8 b	160 b	0.6 b	2.7 b	40 a	37 b
Microshoots	2 c	140 b	0.7 b	3.2 b	30 b	45 a

* Concentrations followed by identical letters are not considered significantly different according to Tukey's range test at $p < 0.05$

seeds. Furthermore, germination rate was inversely proportional to the salt concentration of the medium. This might be explained by the higher negative osmotic potential resulting from a high salt concentration in the growing medium (Erdag et al., 2010).

Cytokinins, especially BA and Kin, are well known inducers of cell division that cause the formation of multiple shoots (Yew et al., 2010). We tested the shoot proliferation of *L. coronopifolia* using MS medium supplemented with different concentrations of BA and Kin. The best medium for the proliferation of microshoots was MS supplemented with 0.5 mg·L^{-1} BA; this resulted in 3.6 shoots per explant. Al-Bakhit et al. (2007) also found MS medium supplemented with 0.5 mg·L^{-1} BA to be the most effective for the proliferation of *L. latifolia* microshoots. Another study in *L. pendunculata* found that the addition of BA to growth medium induced higher numbers of microshoots (Zuzarte et al., 2010). Andrade et al. (1999) found that propagation of *L. vera* microshoots was most effective with a combination of high BA and TDZ hormone.

The response of in vitro-propagated plants is greatly influenced by the balance of endogenous growth regulators, and their interactions with exogenous regulators (Aremu et al. 2014). The present study showed that BA resulted in higher number of shoots than Kin. However, shoot length was longer in plants grown with Kin than with BA. Likewise, *Sphenostylis stenocarpa* shoots were longer when grown in MS media supplemented with Kin versus BA (Adesoye et al. 2012). Al Khateeb et al. (2013) found BA to be superior to Kin for the induction of *Moringa peregrina* shoots, but Kin resulted in longer shoots than BA. BA enhances the production and metabolism of natural endogenous hormones inside plant cells, which stimulate shoot organogenesis (Ahmad and Anis, 2014).

Many studies have shown the induction of roots and root development to be related to auxins (Overvoorde et al., 2010,

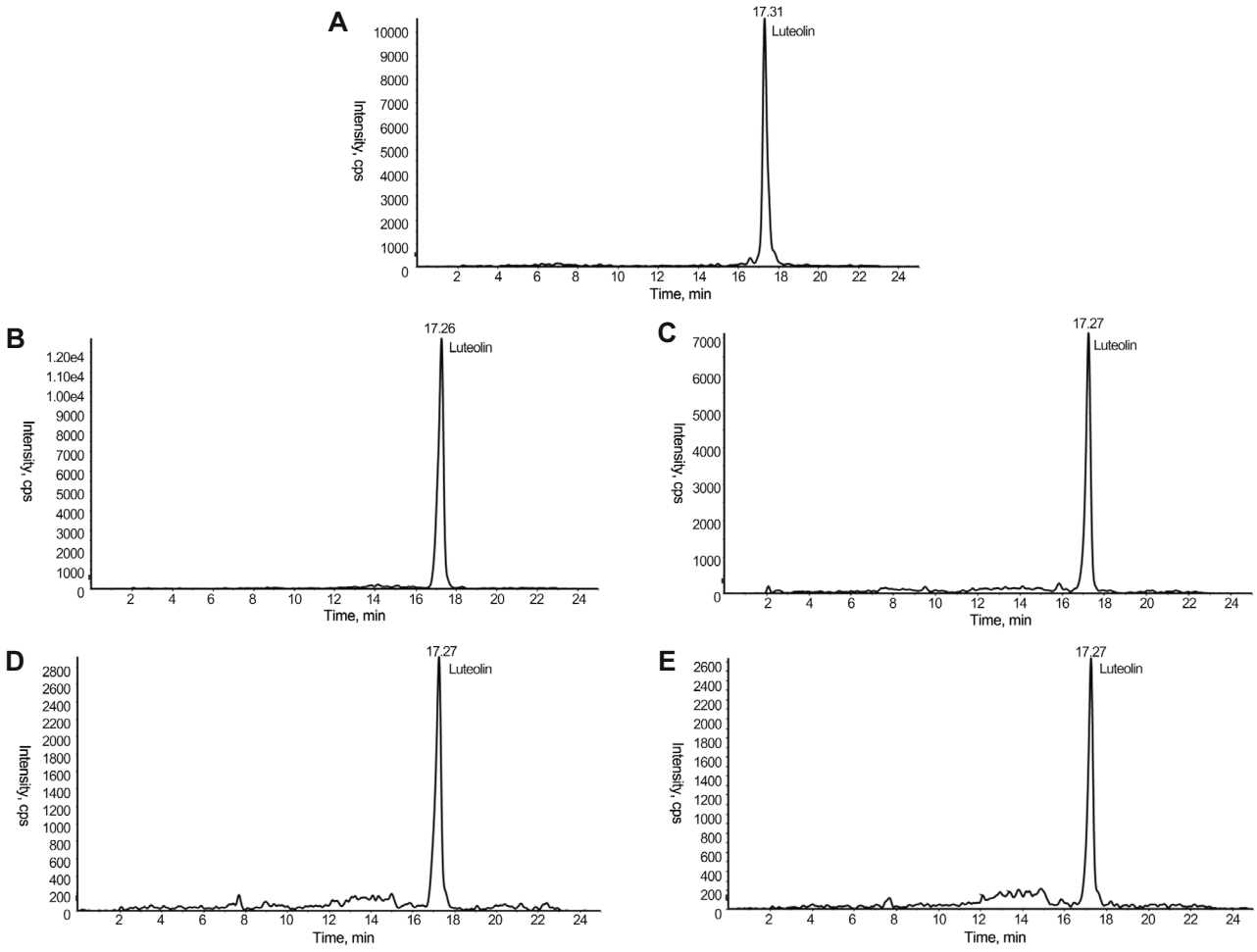

Fig. 8. Multiple reaction monitoring (MRM) ion chromatograms. (A) A reference standard of luteolin, tR = 17.31 min (m/z 285.3 → 132.9); (B) wild *L. coronopifolia*; (C) greenhouse-grown *L. coronopifolia*; (D) *L. coronopifolia* calli; and (E) *L. coronopifolia* microshoots.

Kanaan, 2016). Auxins promote adventitious initiation of roots by increasing the rate of cell division. Falk et al. (2013) found that NAA and IBA induced rooting in many *Lavandula* species; likewise, we found that IBA and NAA induced *L. coronopifolia* roots. A concentration of 1 $mg \cdot L^{-1}$ IBA was the best for root formation. This is in agreement with Sabzevar et al. (2015), who found that 1.0 $mg \cdot L^{-1}$ IBA to be the most effective for root formation in *L. angustifolia*. In contrast, Gonçalves and Romano (2013) and Andrade et al. (1999) found NAA to be the best auxin for root induction for *L. vera*. Abbasi et al. (2013) found that lower rather than higher concentrations of auxin promote root growth because of the production of the growth inhibitor ethylene in the root zone.

The best medium for inducing callus growth in *L. coronopifolia* was a combination of BA and NAA under light conditions - this resulted in large, green, highly friable calli, with the maximum fresh weight of 667.9 mg. Studies of *L. vera* similarly found a combination of BA and NAA to be the best for the formation of friable light green calli (De Silva et al., 2016). However, Falk et al. (2013) reported that MS medium supplemented with TDZ hormone was best for the induction of *L. angustifolia* calli.

We established a growth curve for *L. coronopifolia* by measuring the fresh weight of calli every week for 9 weeks. This curve revealed a sigmoid growth pattern with lag, exponential and stationary phases. The lag phase is a period during which energy stores are produced, which starts at the beginning of culture and lasts until the second week. The exponential phase between weeks 2 and 7 is when maximum cell division occurs. The final phase, the stationary phase (weeks 7-9), is when secondary metabolites accumulate in the tissues and no cell division occurs. This growth curve differed in shape to that of *L. angustifolia*, which showed a logarithmic sigmoidal shape over a period of 4 weeks. Here, the lag phase lasted for 2 weeks, the exponential phase took place between weeks 2 and 3, and the stationary phase continued beyond 4 weeks (De Bona et al., 2012).

Micropropagation is a valuable tool for plant regeneration, but it carries the risk of inducing genetic variability. Growth

regulator type, the number of subculture passages, explant source, culture duration, genetic make-up, growth media, and mutations including point mutations, somatic recombination, sister chromatid exchange, transposable elements and epigenetic processes, are all factors capable of inducing genetic variability for in vitro-propagated plants (Werner et al., 2015). This propensity for genetic variability means a tool is needed to detect somaclonal variations in in vitro-grown plants (Saha et al., 2016). In our study, ISSR-PCR was used as a molecular marker to assess genetic integrity in micropropagated *L. coronopifolia* plants. We found that successive in vitro subcultures of *L. coronopifolia* microshoots had high genetic stability, and the banding patterns indicated close relatedness between mother plants and their in vitro-propagated microshoots. Similarly, Lata et al. (2010) used ISSR banding patterns to confirm the genetic stability of micropropagated *Cannabis sativa* plants and mother plants.

Phytochemical Analysis and Biological Evaluation of *L. coronopifolia*

In this study, we assessed the phytochemical profiles of wild, in vitro-grown microshoots and calli of *L. coronopifolia*. Phenolics are the largest group of phytochemicals with a wide range of biochemical activities (Balakrishnan et al., 2015). Wild plants had double the amount of phenolics (4.9 mg GAE) compared to microshoots, and twice the radical scavenging activity (85%) compared to calli. Other studies have obtained similar results; for example, Costa et al. (2012) found that the total phenolic content and antioxidant activities of wild-grown *Thymus lotocephalus* was greater than in in vitro-grown plants. Another comparative study found that wild-grown *Arnica montana* plants had greater for radical-scavenging activity and polyphenolic content than in extracts of *in* vitro-grown samples (Nikolova et al., 2013). However, García-Pérez et al. (2012) found that in vitro-grown *Poliomintha glabrescens* had higher levels of phenolic compounds and greater antioxidant activity than wild plants.

In general, in vitro-grown plants are exposed to less stressful conditions than wild-grown plants (Chirinea et al., 2012). Differences in phenol content depends on growth conditions and the stage of plant development. Secondary metabolite synthesis increases under natural conditions as a defense mechanism against environmental stress.

Assessment of cytotoxic lethality is essential to determine the effectiveness of medicinal plants in traditional medicine. We used a brine shrimp bioassay to assess the cytotoxicity of methanolic extracts of wild *L. coronopifolia* plants, microshoots, and calli. According to Clarkson's classification, herbal plant extracts with an LC_{50} above 1000 $\mu g \cdot mL^{-1}$ are considered nontoxic, those with an LC_{50} of 500-1000 $\mu g \cdot mL^{-1}$ are considered to have low toxicity, and those with an LC_{50} of 100-500 $\mu g \cdot mL^{-1}$ have medium toxicity (Hamidi et al., 2014). The *L. coronopifolia* extracts assayed in this study were considered nontoxic. Another study found that *L. dentate* has low toxicity (Aly et al., 2013), while Gadir (2012) reported no cytotoxic activity for *Ocimum basilicum* plants with an LC_{50} >1000.

We also assessed the biological phytotoxic effects of *L. coronopifolia* extracts on the germination of radish (*Raphanus sativus*) seeds and their effect on the length of radical growth. Wild plant extracts were the most highly toxic to radish seeds, where a concentration of 0.6 $mg \cdot mL^{-1}$ reduced the germination percentage to 70% and radical length to just 1.1 cm. Callus extract was the least toxic, with 90% radish seed germination and an average radical length of 3.5 cm. In a study of the toxicity of different members of the *Lamiaceae* family on radish seeds, De Almeida et al. (2010) found that oregano, thyme, sage, and lavender inhibited germination and radical elongation by 100% at high doses.

The *Lamiaceae* family is known to have potent natural antioxidant activity because of the presence of various phenolic compounds that act as a defense system. Most *Lavandula* species exhibit biological activity because of the presence of phenolic compounds such as caffeic, chlorogenic and rosmarinic acids, quercetin, and luteolin (Messaoud et al., 2012). Using LC-MS, we identified and quantified six major phenolic compounds in the crude extracts of *L. coronopifolia*, namely caffeic acid, rosmarinic acid, rutin, quercetin, luteolin, and hesperidin. In vitro-grown microshoots and calli had the highest concentrations of caffeic acid and rosmarinic acid, respectively. These data suggest that in vitro propagation can enhance the production of these phenolic compounds, indicating the potential for their optimization in future studies. Caffeic and rosmarinic acids are known to be potent antioxidants, inhibiting DNA methylation, and having cardioprotective and antimicrobial effects (Bubenchikova et al., 2014). However, the crude sample of callus extract enhanced the production of all phenolic compounds to a greater extent. Furthermore, our qualitative analysis revealed six major peaks in crude in vivo and in vitro samples, with the same retention times and different peak areas, whereas *in vitro*-grown samples had higher peak areas than the wild plant samples. This suggests that the in vitro propagation protocol increased the production of these compounds.

Using LC-MS, Moldovan et al. (2014) identified and quantified five major phenolic compounds in *Mentha* species (another genus in the *Lamiaceae* family): caftaric acid, gentisic acid, caffeic acid, chlorogenic acid, and p-coumaric acid. Another *Lavandula* study identified eight phenolic compounds: 2-O-glucosilcoumaric acid, O-coumaric acid, rosmarinic acid, apigenin, luteolin, herniarin, coumarin, and apigenin-7-O-glucoside (Areias et al., 2000). Al Khateeb et al. (2017) found that the addition of NaCl and mannitol to the growing medium caused the production of new phenolic

compounds (gallic acid and chlorogenic acid) in *Rumex*.

The present study describes, for the first time, a successful in vitro propagation protocol for *L. coronopifolia* and reveals that MS medium supplemented with 0.5 mg·L^{-1} BA is the best medium for shoot proliferation. For root formation however, MS medium supplemented with 1.0 mg·L^{-1} IBA was best, and for the formation of calli, MS medium supplemented with 1.0 mg·L^{-1} NAA + 0.5 mg·L^{-1} BA was the best. The *L. coronopifolia* callus growth curve revealed an S-shaped growth with optimal growth between weeks 2 and 4. Total phenolic content and antioxidant activity was higher in wild-grown plants than in vitro-grown plants. Extracts of wild *L. coronopifolia* plants, microshoots and calli are nontoxic.

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