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ORIGINAL PAPER

In vitro morphogenetic responses and comparative analysis of phthalides in the highly valued medicinal plant *Ligusticum porteri* Coulter & Rose

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Abstract The morphogenetic response of *Ligusticum porteri,* a medicinal and ceremonial plant, was investigated as part of the conservation strategy of this wild species and was compared to that of a cultivated species, *Petroselinum crispum*. Seeds were germinated in half strength Murashige and Skoog medium. Plantlets were excised into root, cotyledon, petiole, stem and leaf explants and cultured in an induction medium supplemented with the range of 0–18.09 μM 2,4-dichlorophenoxyacetic acid (2,4-D) or 0–21.48 μM α-naphthaleneacetic acid in combination with 0–13.31 μM 6-benzylaminopurine. Calli derived from leaf, seeds, petiole, stem and roots, mature aerial parts and roots extracts of *L. porteri* and *P. crispum* were analyzed by thin layer chromatography and gas chromatography coupled to mass spectrometry. 3-Butylidenephthalide (6.3%) was identified along with other 23 compounds from mature aerial parts extract of *L. porteri* and also in its roots (20.8%). 3-*n*-Butylphthalide (0.7%) and 3,6,7,-trimethoxy-isobenzofuran-13(H)-one (4.9%) were identified from the roots of *P. crispum.* 3-Butylidenephthalide was identified from two petiole (0.9%; 0.26%) and one stem (0.8%) callus extracts of *L. porteri.* This is the first report on phthalides production from in vitro cultures of *L. porteri*. The results indicated that in vitro cultures of this plant possess the biosynthetic machinery for the biosynthesis of these highly valuable compounds.

Keywords *Ligusticum porteri* · *Petroselinum crispum* · Apiaceae · Callus culture · Phthalides

Abbreviations

MS	Murashige and Skoog
2,4-D	2,4-Dichlorophenoxyacetic acid
NAA	α-Naphthaleneacetic acid
BA	6-Benzylaminopurine
GA_3	Gibberellic acid
TLC	Thin layer chromatography
GC–MS	Gas chromatography coupled to mass spectrometry
TTZ	2,3,5-triphenyl-2H-tetrazolium chloride
PPM	Plant preservative mixture
PCV	Packed cell volume

Introduction

Ligusticum porteri Coulter & Rose (Apiaceae), "chuchupate", is a perennial herb that produces a thick vertical rootstock with crown of vascular bundles from decayed petioles at the top and a strong characteristic odor (Linares and Bye 1987). It grows in pine-oak forests of the southern and central Rocky Mountains of the United States and the northern Sierra Madre Occidental of Mexico (Cronquist 1997).

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In the Mexican folk medicine, *L. porteri* is highly prized for medicinal and ceremonial purposes by Tarahumara, Pimas, Zuni, Pauite and Mescalero Apache people (Linares and Bye 1987; Camazine and Bye 1980). Concoctions of the root are employed in the treatment of gastrointestinal disorders, such as stomachache, colic, ulcer and diarrhea, as well as headache, fever, cold, sore throat, wound, rheum, pain, bronchitis, pneumonia, tuberculosis and scorpion bites (Linares and Bye 1987). Portions of the root are used as a talisman to ward off witches and prevent snakebites as well as a good luck charm (Appelt 1985; Ortíz et al. 2007; Bye 1985).

Phthalides are reported to be the biologically active compounds responsible for the therapeutic action of this species. They have smooth muscle relaxant activity (Du et al. 2006), vasorelaxing activity (Chan et al. 2006), analgesic, neuroprotective (Peng et al. 2007; Kuang et al. 2006), antisclerotic, antihypertensive (Lu et al. 2006), serotonergic (Deng et al. 2006a), GABAergic (Deng et al. 2006b), antiproliferative (Liang and He 2006), antihyperglycemic (Brindis et al. 2011), sedative and spasmolytic (León et al. 2011). These compounds have been found in some genera of the Apiaceae including *Angelica* (Lao et al. 2004), *Anethum* (Fischer and Gijbels 1987), *Apium* (MacLeod et al. 1988), *Bifora* (Gijbels et al. 1985), *Capnophyllum* (Gijbels et al. 1984), *Cnidium* (Gijbels et al. 1981), *Conioselinum* (Gijbels et al. 1979), *Levisticum* (Santos et al. 2005), *Ligusticum* (Delgado et al. 1988), *Lomatium* (Bairamian et al. 2004), *Opopanax* (Gijbels et al. 1983), *Petroselinum* (Gijbels et al. 1985), *Peucedanum* (Gijbels et al. 1984) and *Meum* (Palá-Paúl et al. 2004).

Because of the overexploitation of its roots to satisfy the increasing demand of these molecules and the reduction of natural populations due to habitat destruction, a conservation strategy for *L. porteri* is highly required. Conventional cultivation has been hindered because of its asynchronous and low rate of seed germination. In vitro germination and micropropagation provide biotechnological alternatives for the production of plants for forest revegetation as well as in vitro synthesis of bioactive compounds. A first step in this process is to establish and evaluate the in vitro morphogenetic responses of *L. porteri*. We undertook this study because little is known about its natural reproduction and nothing has been reported about its morphogenetic and biosynthetic responses under in vitro conditions. Results were compared to those of *Petroselinum crispum* (Mill.) Nyman ex A.W. Hill, a cultivated species of the Apiaceae, that also biosynthesizes phthalides. Finally, the presence of phthalides in the root and callus extracts of *L. porteri* and *P. crispum* was assessed by thin layer chromatography (TLC) and gas chromatography coupled to mass spectrometry (GC–MS).

Materials and methods

Plant material

Mature seeds (technically modified fruits known as mericarps) and leaves of *L. porteri* were collected from wild plants in the pine-oak forests near Norogachi, Municipio of Guachochi, Chihuahua, Mexico, in October of 2005 (specimen voucher BYE 34467). After cleaning the seeds, they were stored at 1°C for 45 days. In the case of *P. crispum,* seeds of the commercial brand "Semillas Vita" (Rancho Los Molinos S. P. R. de R. L. Morelos, Mexico) were purchased from a local market. Seed viability was tested using the 2,3,5-triphenyl-2H-tetrazolium chloride (TTZ) conventional technique (Schmidt 2000).

Ex vitro germination

Cold pretreated and not-pretreated seeds of *L. porteri* were placed in pots (30 × 30 cm) with different mixtures of substrates: (a) *Sphagnum* and soil (1:1 v/v), (b) agrolite, (c) soil and agrolite (1:1 v/v) and (d) soil. These were kept in greenhouse conditions.

In vitro germination

In order to obtain axenic seedlings of *L. porteri* and *P. crispum*, the surface disinfected seeds were germinated in 100 mL jars containing 25 mL of a half strength Murashige and Skoog (1962) medium with 30 g L^{-1} sucrose, which was solidified with 4 g L^{-1} Gelrite (w/v) (*Phyto*Technology Laboratories®, Shawnee Mission, KS, USA). The pH of the medium was adjusted to 5.7 prior autoclaving at 120°C and 103 kPa for 17 min. The pericarp was removed with tweezers after 1 h stirring the seeds in tap water. Surface disinfection was carried out by subjecting sequentially the seeds to the following process: tap water and commercial detergent for 20 min, a solution of hydrogen peroxide (7% v/v) for 7 min, a solution of terramycin (2 g L^{-1}) for 10 min, a solution of cuprimicin (3 g L^{-1}) for 10 min, a solution of sodium hypochlorite (domestic bleach 10% v/v) for 10 min and, finally, a solution of Plant Preservative Mixture™ (PPM) (Plant Cell Technology, Washington, DC, USA) (20.4 mL L^{-1}) for 2 h. Seeds were incubated at 21°C in darkness and after germination transferred to fluorescent light (13.5 μmol m^{-2} s^{-1}) with a 16 h light and 8 h dark photoperiod.

Callus induction media and cell cultures

10–20 weeks old plantlets of *L. porteri* and *P. crispum* were excised to produce cotyledon, hypocotyl, stem, root, leaf

and petiole explants that were cultured in a half strength Murashige and Skoog (MS) medium and 30 g L^{-1} sucrose supplemented with 2,4-D (0, 0.45, 1.81, 4.52, 9.05, 13.57, and 18.09 μM) in combination with benzylaminopurine (BA) (0, 2.22, 4.44, 8.87, and 13.31 μM) or α-naphthaleneacetic (NAA) (0, 0.54, 2.69, 5.37, 10.74, 16.11, and 21.48 μM) in combination with BA (0, 2.22, 4.44, 8.87, 13.31 μM) and incubated at 21°C under a photoperiod for 30 days. To develop suspension culture, callus forming explants were transferred to a half strength liquid MS media with their respective plant growth regulator treatment.

Growth kinetics and determination of fresh weight

A series of 20 glass flasks of 100 mL volume, each containing 25 mL of culture medium, were inoculated individually with 5 mL of an established cell suspension culture and allowed to grow for 30–90 days in a rotary shaker (90 rpm) at 21°C. The growth was terminated on alternate days by taking 3 aliquots of 1 mL and placed in 1.5 mL sterile Eppendorf tubes. The suspension growth was measured by packed cell volume (PCV) (Street 1977). Each sample tube was centrifuged in an Eppendorf 5415D centrifuge at 2,600 rpm for 3 min and then the PCV was weighed in an analytic balance. Different stages of the growth curve were analyzed with a photomicroscope Axoskop C Zeiss both in clear and contrast phases. Samples were stored in the dark at 1°C.

Extraction of plants

Material derived from roots (1.52 g), aerial parts (126 g) of *L. porteri* and roots of *P. crispum* (4.30 g) from greenhouse and field collections were powdered and macerated (500 mL) with a mixture of methylene chloride-methanol (analytical grade) 1:1 v/v at room temperature for 7 days in complete darkness. The resulting extracts were concentrated by distillation in a vacuum rotary evaporator at 60°C to yield a 0.17, 11.37 and 0.38 g crude extracts, respectively. All extracts were analyzed by TLC with *Z*-ligustilide (**1**) and diligustilide as standard references. All extracts were further analyzed by GC-MS. The aerial parts extract was subjected to silica gel column chromatography (250 g), eluting with a hexane–ethyl acetate (analytical grade) gradient (10:0 → 0:10). The eluents were combined to yield eight fractions on the basis of TLC analysis. Fractions I, II, III-A, III-B, IV, V, VI and VII were further analyzed by GC–MS.

Extraction of calli

Material derived from in vitro callus cultures of root (32 mg), cotyledon (28 mg), seeds (7 mg), leaf (228.5 mg), root (50.2 mg), petiole (270.5 mg), stem (49 mg), petiole (136 mg), root (53 mg) and petiole (52 mg) were processed as mentioned above and analyzed by TLC and GC–MS. Treatment conditions are listed in Table 1.

TLC assay

A 1 μL aliquot of extracts were spotted on silica-gel aluminum plates (F254, 10 × 20 cm × 0.25 mm, Merck, Germany) and developed with the mobile phase with toluene:ethyl acetate:acetic acid 90:10:1 v/v/v. A 1 μg of *Z*-ligustilide was spotted as positive standard.

GC–MS conditions

GC–MS analyses were carried out using an Agilent Technologies 6890 N chromatograph with a split injector (split relation 1/50) coupled to a LECO Pegasus 4D mass spectrometer. The MS detector was used in the electron impact ionization (EI) with an ionization voltage of 70 eV, using a total swept of spectra from 45 to 800 m/z. The

Table 1 Culture conditions of the analyzed calli of *L. porteri*

Explant	Plant growth regulator combination
Cotyledon	2,4-D (13.57 μM)/BA (4.44 μM)
Leaf	2,4-D (18.09 μM)/BA (4.44 μM)
Petiole I	2,4-D (18.09 μM)/BA (2.22 μM)
Petiole II	2,4-D (0.45 μM)/BA (4.44 μM)
Petiole III	2,4-D (0.45 μM)/BA (13.31 μM)
Root I	2,4-D (13.57 μM)/BA (4.44 μM)
Root II	NAA (5.37 μM)/BA (4.44 μM)
Root III	NAA (21.48 μM)/BA (8.87 μM)
Seeds	GA_3 (28.89 μM)
Stem	2,4-D (0.45 μM)/BA (13.31 μM)

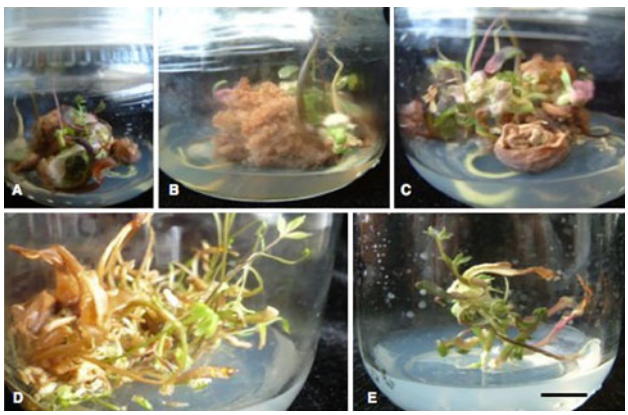

Fig. 1 Seeds of *L. porteri* germinated in vitro. **a–c** Seeds pretreated with GA_3 (28.89 μM) that developed into callus and the regeneration of a new plant. **d–e** Seeds with no GA_3 pretreatment with direct organogenesis. *Barr*: 3 cm

silica semi polar capillary column (DB-5MS; 5% phenyl 95% methylpolysiloxanes; 10 m length × 0.18 mm internal diameter × 0.18 μm film thickness) was used with He as carrier gas with a constant flux of 1 mL/min. The injector temperature was 300°C. The transference line temperature was 250°C and the source of ionization was 200°C. The mass spectra were deconvoluted by AMDIS® (NIST, Gaithersburg, MD, USA) software and the compounds identified by comparing their mass spectral fragmentation and retention time with those of reference compounds spectra from NIST 05 database [NIST Mass Spectral Database, PC-Version 5.0 (2005) National Institute of Standardization and Technology, Gaithersburg, MD, USA], standard references compounds and literature.

Results and discussion

Seed viability test

TTZ seed viability test indicated that only 50% of seeds of *L. porteri* maintained at 10°C and −10°C for 45 days were viable. Seed viability results increased to 100% when seeds were stored at 1°C for 45–90 days.

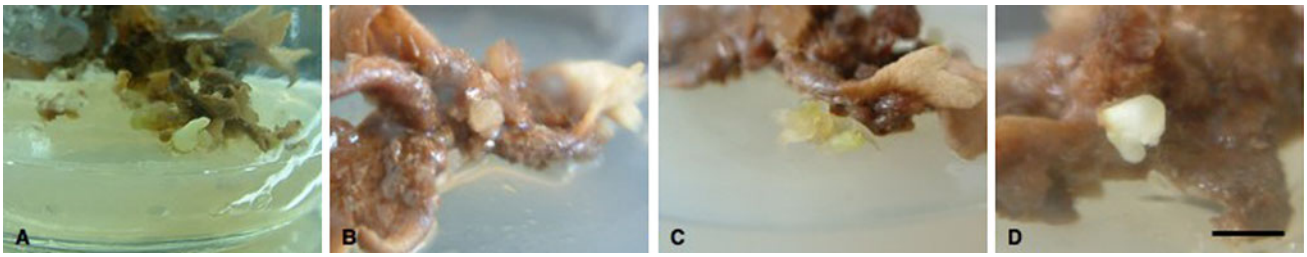

Fig. 2 Morphogenetic leaf of *L. porteri* cultured with 2,4-D (9.05 μM) and BA (4.44 μM). **a** Somatic embryo. **b** Cotyledonary-like stage embryo. **c** Nodular structures. **d** Pear-like structure embryo with a suspensor. *Barr*: 2 cm

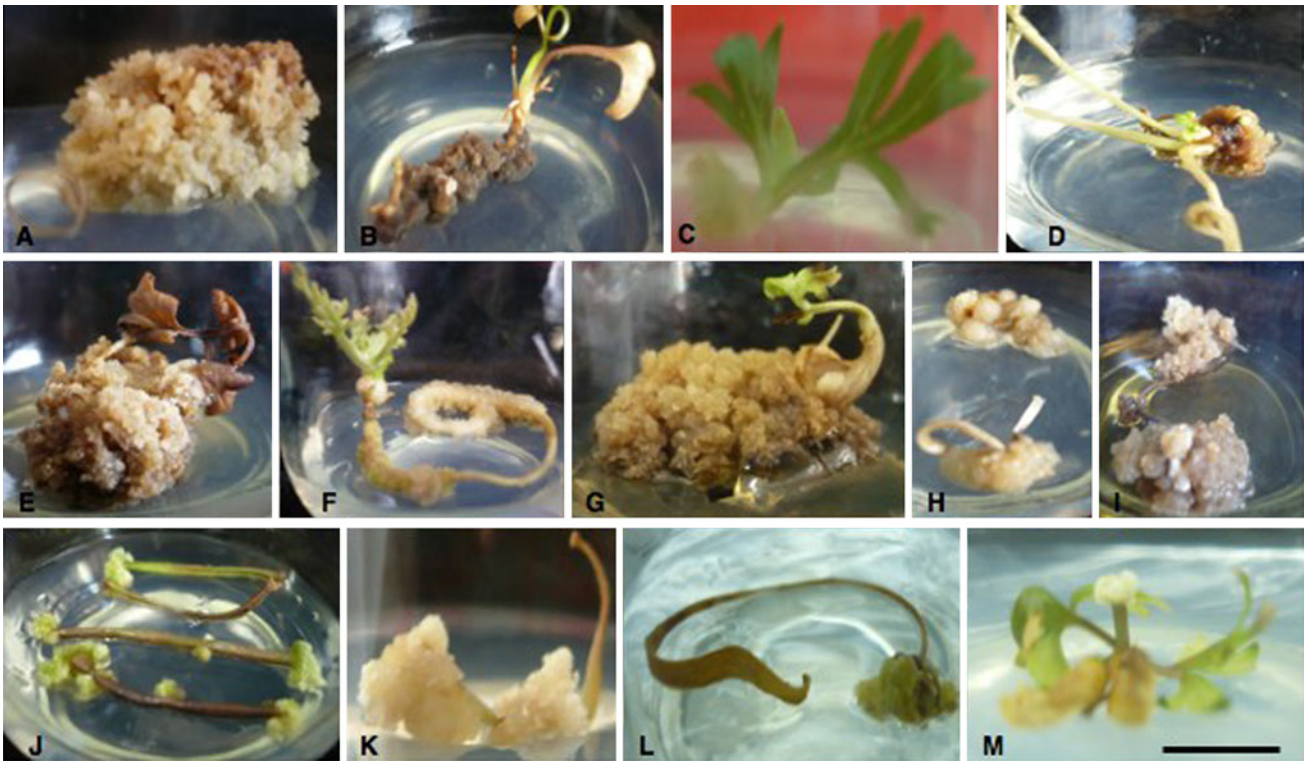

Fig. 3 Morphogenic responses of all explants of *L. porteri.* **a** Cotyledon NAA (16.11 μM)/BA (4.44 μM), **b** root and stem NAA (16.11 μM)/BA (4.44 μM), **c** leaf NAA (16.11 μM)/BA (4.44 μM), **d** stem 2,4-D (0.45 μM)/BA (4.44 μM), **e** leaf 2,4-D (18.09 μM)/BA (4.44 μM), **f** root and stem 2,4-D (4.52 μM)/BA (4.44 μM), **g** stem 2,4-D (18.09 μM)/BA (8.87 μM), **h** Cotyledon 2,4-D (2.26 μM), **i** petiole NAA (21.48 μM), **j** petioles 2,4-D (0.45 μM)/BA (4.44 μM), **k** petiole 2,4-D (13.57 μM)/BA (4.44 μM), **l** cotyledon 2,4-D (0.45 μM)/BA (2.22 μM), **m** leaf NAA (0.54 μM)/BA (13.31 μM). *Barr*: 1.5 cm

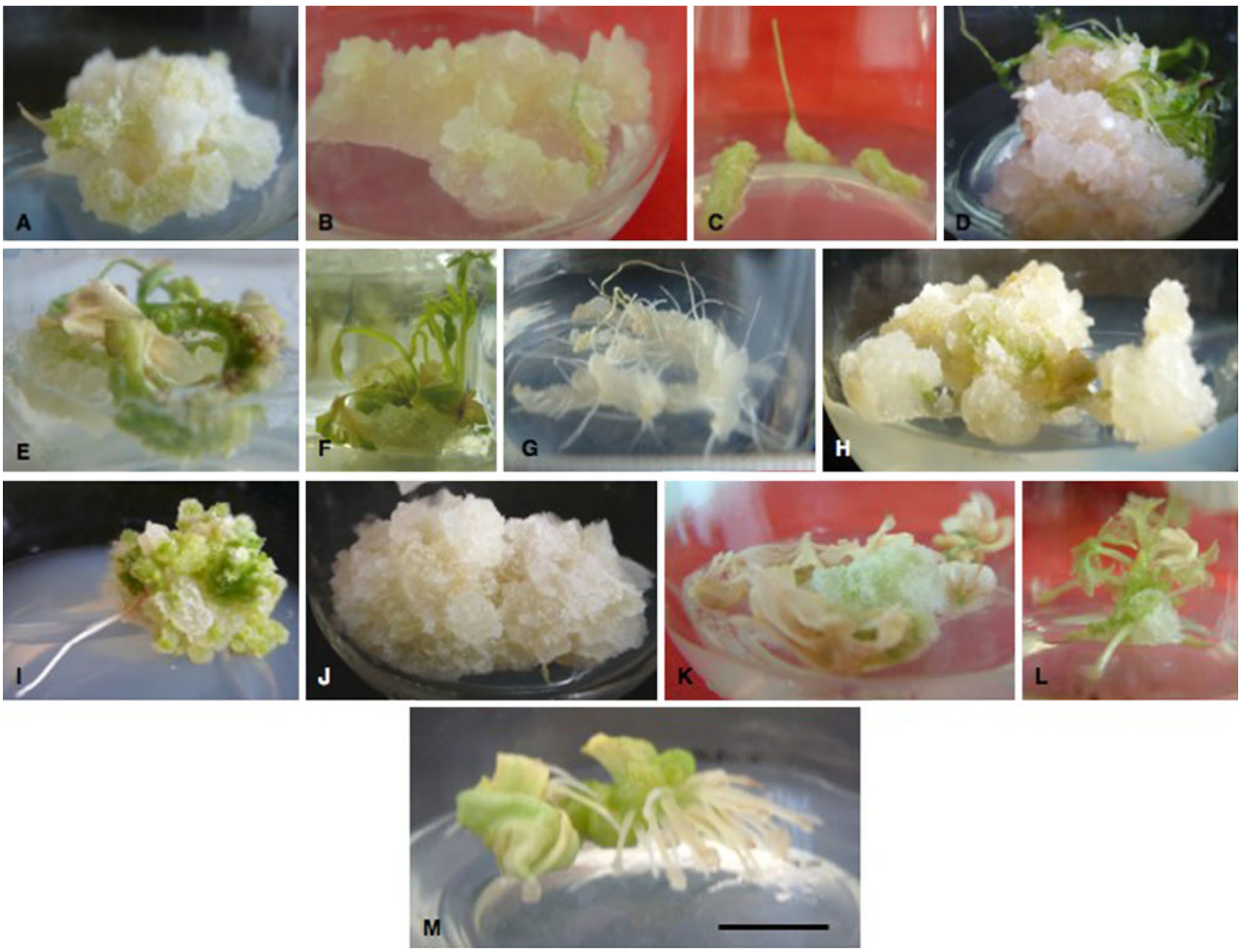

Fig. 4 Morphogenic responses of all explants of *P. crispum.* **a**. Leaf NAA (21.48 μM)/BA (4.44 μM), **b** root 2,4-D (4.52 μM)/BA (4.44 μM), **c** root and stem NAA (21.48 μM), **d** petiole NAA (16.11 μM)/BA (2.22 μM), **e** cotyledon NAA (21.48 μM)/BA (2.22 μM), **f** leaf NAA (0.54 μM)/BA (2.22 μM), **g** root NAA (21.48 μM)/BA (4.44 μM), **h** petiole 2,4-D (0.45 μM)/BA (4.44 μM), **i** cotyledon 2,4-D (9.05 μM), **j** leaf BA (4.44 μM), **k** leaf 2,4-D (9.05 μM)/BA (4.44 μM), **l** stem NAA (10.74 μM)/BA (4.44 μM), **m** leaf NAA (21.48 μM). *Barr*: 1.5 cm

Ex vitro germination

The three substrates [agrolite, *Sphagnum* and soil (1:1:1 v/v/v); agrolite and soil (1:1 v/v); and agrolite] did not influence germination of *L. porteri* seeds. In the case of seeds without cold pretreatment, the germination was low (5%) and all the seedlings died after 12 weeks. Germination was higher (95%) in the case of the seeds stored at 1°C for 45 days and developed after 3–4 weeks regardless the substrate; the seedlings had a 100% survival rate. These plantlets had a healthy aspect, a fully expanded and green leaf and a long and thick root.

In vitro germination

Seeds of *L. porteri* pretreated with 28.89 μM of gibberellic acid (GA_3) for 24 h did not germinate. Instead, 1.5% of

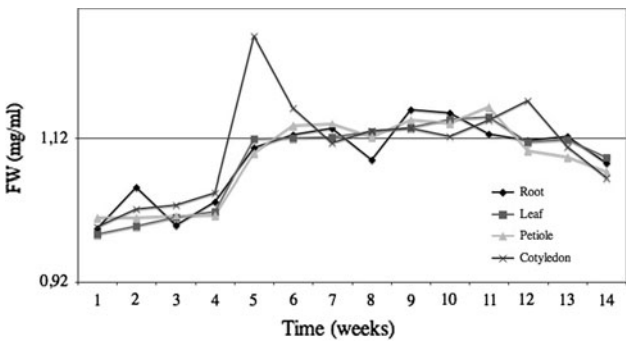

Fig. 5 Growth kinetics of all calli of *L. porteri*

embryos developed into callus and structures that resembled products of embryogenesis. They produced multiple petioles, axillary buds, curved adventitious-like buds,

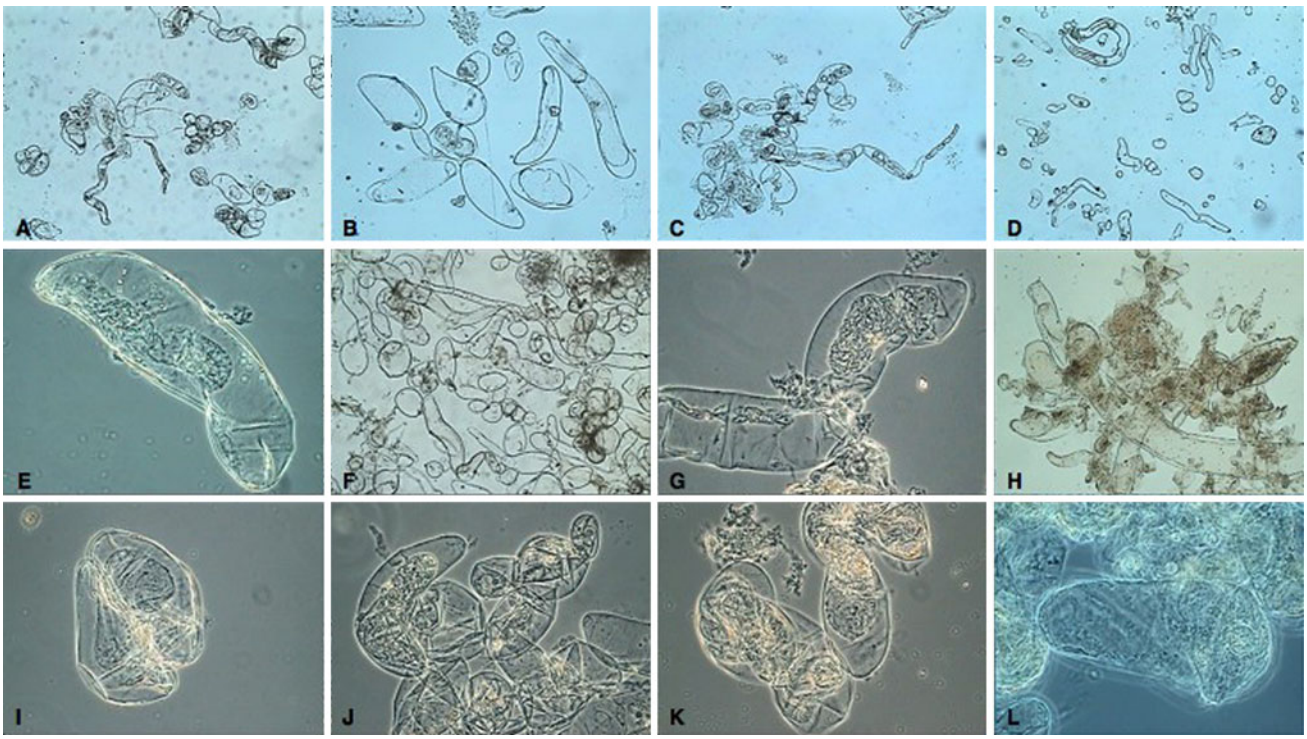

Fig. 6 Photomicrography of cell suspension cultures of *L. porteri*. **a** Cotyledon ×1,000 cp, **b** leaf ×2,000 cp, **c** petiole ×100 cp, **d** root ×100 cp, **e** cotyledon ×100 cp, **f** leaf ×200 cp, **g** petiole ×400 cp, **h** root ×200 cp, **i** cotyledon ×400 cp, **j** leaf ×400 cp, **k** petiole ×400 cp, **l** root ×1,000 cp. *n* nucleus, *cc* contracted cytoplasm

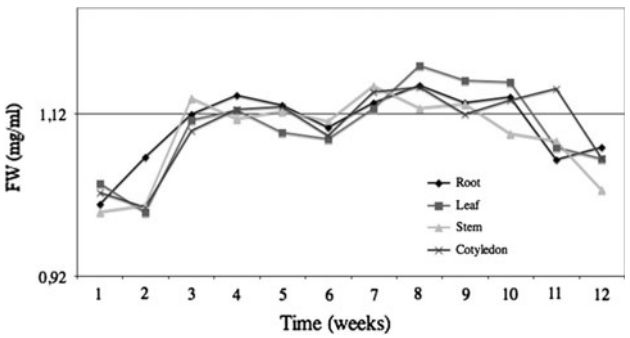

Fig. 7 Growth kinetics of all calli of *P. crispum*

a robust root, enlarged, concave and thick cotyledons and a large biomass in a free of growth regulators MS medium (Fig. 1a–c). This demonstrates that GA_3 did not substitute cold treatment as reported for other species in the same family such as *Chaerophyllum temulum* (Vandelook et al. 2007) and *Ferula gummosa* (Nadjafi et al. 2006). The seeds of *L. porteri* with cold pretreatment germinated after five weeks of incubation in half strength MS media while those of *P. crispum* germinated in 2 weeks. The seeds of *L. porteri* germinated asynchronously (ca. 2%/week) after 5 weeks of incubation and the seedlings presented low survival (50%) after 6 weeks. In contrast at the end of 2 weeks, *P. crispum* germinated synchronously and all the seedlings survived.

Seeds of *L. porteri* cultured in a free of growth regulators MS medium showed a direct morphogenesis with organogenesis producing foliar, adventive and axillary buds. Plantlets had fully expanded leaves, stems, roots and sometimes cotyledon-like structures (Fig. 1d, e). These structures have a regenerative potential regardless of growth regulators in the medium. These results also revealed the high polymorphism of seeds as a result of a genetic recombination or the reaction to the in vitro conditions of a wild species.

Plantlet decontamination

Explants from plantlets of *L. porteri* grown *ex vitro* were difficult to establish aseptically in vitro. Thirteen combinations including PPM, amoxicillin, gentamicin (Salehi and Khosh-Khui 1997), terramycin, benomyl (Hauptmann et al. 1985) cuprimicin and thiabendazole (Tecto 60) were added to the media. Even though the combinations of fungicides and a mixture of all reagents were able to control the fungal contamination, all the explants died after 3 weeks. Consequently, plantlets derived from *ex vitro* germination were discarded.

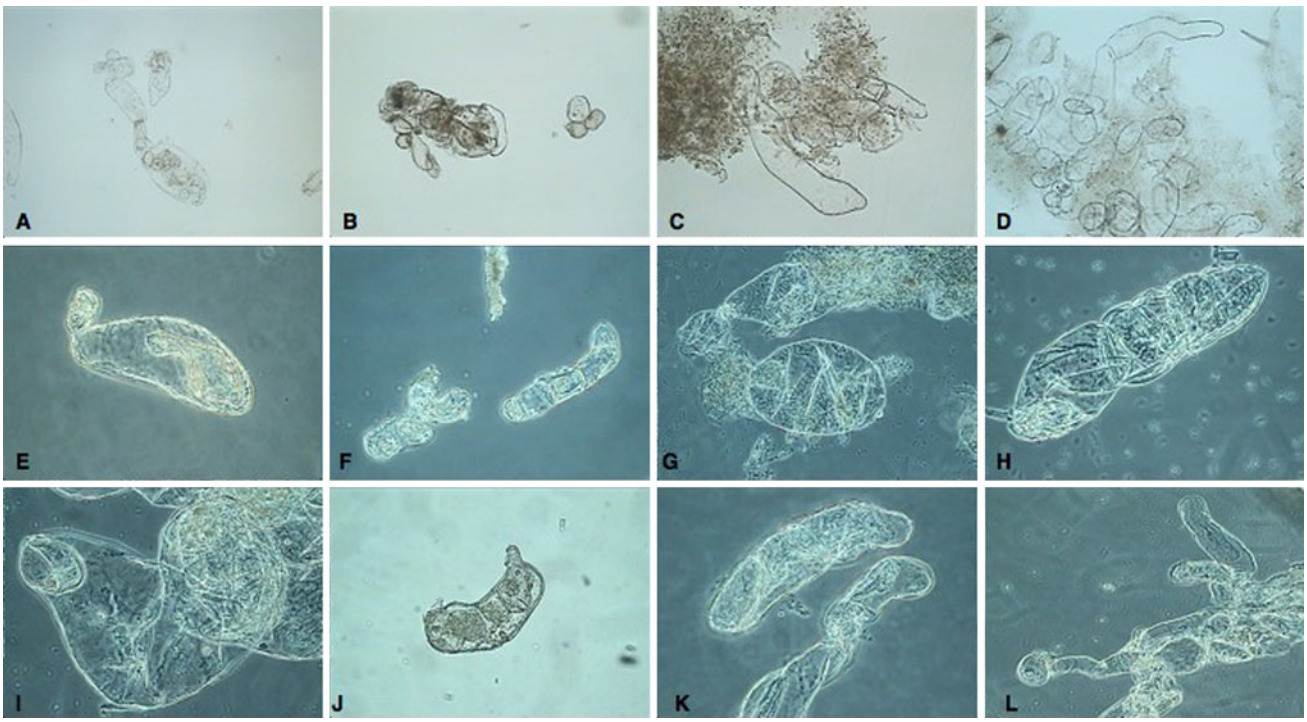

Fig. 8 Photomicrography of cell suspension cultures of *P. crispum*. **a** cotyledon ×100 cp, **b** leaf ×200 cp, **c** root ×200 cp, **d** stem ×200 cp, **e** cotyledon ×400 cp, **f** leaf ×400 cp, **g** root ×200 cp, **h** stem ×400 cp, **i** cotyledon ×400 cp, **j** leaf ×400 cp, **k** root ×400 cp, **l** stem ×200 cp. *cn* cellular content, *cc* contracted cytoplasm

Morphogenetic cultures

Only 2% of explants derived from leaf, root and stems of *L. porteri*, after induction to callus, resulted in the regeneration of a complete plantlet. Leaf explants cultured in a half strength MS medium supplemented with 2,4-D (9.05 μM) and BA (4.44 μM) developed into a white somatic embryo-like organized structures. They resembled an embryo in the starch storage stage and developed hyaline nodules, roots, with a pear-like morphology and a short suspensor (Fig. 2a–d). They all developed from callus, resulting from an indirect morphogenesis.

Callus induction

Due to the low survival of *L. porteri* plantlets germinated in vitro, it was difficult to explore their response to different combinations of plant growth regulators. More than 80% of explants cultured in a half strength MS medium supplemented with 2,4-D (9.05 and 13.57 μM) and BA (4.44 μM) developed into a yellowish, compact and non-friable callus in a period of 2–3 months (Fig. 1k). Limited growth was due to basal oxidization that progressed into the rest of the tissue. This oxidization was not lethal because the callus continued to grow slowly. After 30–40 days of induction, 90% of these explants became necrotic and died. About 20% of explants developed into callus. Concentrations of NAA (16.11 μM) and BA (4.44 μM) resulted in a faster morphogenetic response. However, 90% of explants died and a lower quantity of callus were obtained compared to that of 2,4-D (4.52–18.09 μM). Explant induction at concentrations of NAA (21.48 μM) and BA (4.44 μM) occurred after 1 month of culture but 95% explants died. This callus developed secondary roots and leaves with extended petioles regardless of the origin of the explant (Fig. 3b, d, f). In the case where the stem and root were intact in the explant, leaves developed from the stem portion. On the other hand, survival of *P. crispum* plantlets was 100% and all explants that were cultured with 2,4-D (0–18.09 μM) or NAA (0–21.48 μM) in combination with BA (0–13.31 μM) developed into a green or hyaline, loose, wet and highly friable callus in less than 30 days (Fig. 4). The combination that gave rise to the highest number of callus was NAA (21.48 μM)/BA (4.44 μM) and 2,4-D (18.09 μM)/BA (4.44 μM). Leaf and root explants cultured with high auxin concentrations developed secondary hyaline, thin roots (Fig. 4g, m). After this time, all callus from both species was cut into 2–3 cm^3 pieces and transferred to 25 mL a half strength liquid MS media, under which they proliferated rapidly. Over 90% of the explants of the wild species became necrotic as auxin concentrations increased and the remainder developed into small to medium size, hard, yellowish callus; in contrast all the explants of the

cultivated species produced a lot of friable, green-transparent callus regardless of the concentration of plant growth regulators.

Growth kinetics

The growth curves of *L. porteri* calli illustrate a typical sigmoid pattern (Fig. 5). Lag phase had no cell division signal (Fig. 4a–d), cellular morphology was elongated primarily and 1–2 mm aggregates and isolated cells were observed. Some cells presented an expanded (Fig. 6b, c) or contracted (Fig. 6i) cytoplasm. Cellular contents (Fig. 6e–h) and nuclei (Fig. 6k) were visible in the later phases.

In the case of *P. crispum* (Fig. 7), all explants, except the root culture, exhibited a similar behavior. In the sixth week the culture conditions may not have been appropriate or the factors became limiting because all explants decreased notably in biomass (0.8 mg mL^{-1} FW After the seventh week all explants increased their biomass (1.4 mg mL^{-1} FW but declined after that. The cells enlarge in size as well as their vacuole (Fig. 8a–c). Clustered (Fig. 8E) or solitary (Fig. 8d) cells as well as cellular content were visible (Fig. 8h, j).

Extract analysis

Eight-month-old roots of *L. porteri* and *P. crispum* (Table 2) cultivated in greenhouse were analyzed by GC–MS. Analyses revealed the presence of 3-butylidenephthalide (20.85%) (**3**) in the former and 3-*n*-butylphthalide (0.72%) (**4**) and 3,6,7-trimethoxy-isobenzofuran-13(H)-one (4.99%) (**5**) in the latter. Other metabolites like ferulic acid (1.67%) (**6**), 5,7,8-trimethyldihydrocoumarin (6.73%) (**7**), psoralen (2.62%) (**8**), apiol (23.51%) (**9**), 2-methoxy-4-vinylphenol (1.16%) (**10**) and falcarinol (17.56%) (**11**), naturally occurring to the Apiaceae, were also found to be present (Fig. 9).

β-Caryophyllene (34.7%) and germacrene D (65.4%) were the only components in the seeds (9.81 g) of *L. porter*. *E*-butylidenephthalide (6.3%) (**2**) was the only phthalide identified in the extract (fraction III-B) from the aerial parts of *L. porter*. Carvacrol, germacrene D, *β*-bisabolene (33.80%), apiol elemicin, α-spathulenol, oplopanone and scoparone were other common compounds detected in the remaining fractions of this extract (Table 3). It should be noted that this is the first report of the presence of a phthalide in the aerial parts of *L. porteri*.

Previous investigations revealed that the variation in contents of the essential oils between a wild and a cultivated species belonging to the same family is very high, even in the same species, these differences become visible. Staniszewska et al. (2005) found in *Daucus carota* ssp. *carota* and *D. carota* ssp. *sativus* a variation in monoterpenoids and sesquiterpenoids content; in the wild subspecies the monoterpenoids were in high concentrations but carotol, daucol and daucene were not detected. On the other hand, the cultivated subspecies contained a high concentration of carotol and low concentrations of monoterpenoids.

L. porteri and *P. crispum* share some characteristic components such as phthalides and phenylpropanoids. Both species accumulate a wide array of metabolites that play a defense role against several microorganisms and herbivores, which surround its environment. It is possible that the chemical composition of the root of *L. porteri* could change if it is domesticated, although the presence of phthalides is a character that has not been altered over the course of domestication.

Calli analysis. GC–MS analysis of the calli of *L. porteri* revealed that *Z*-ligustilide was not present. Nevertheless, 2-methoxy-4-vinylphenol (0.17–46.92%), thymol (0.06%), apiol (0.53–21.05%), falcarinol (3.01–12.91%), acetovanillone (2.65–3.17%), 4-methoxtcinnamate (7.69–100%)

Table 2 Components in the root extracts of *L. porteri* and *P. crispum* (relative abundance, %)

Component	R.T. (min)	*L.p.* %	*P.c.* %
p-Cimene	1.87	0.32	–
Sabinene	1.91	0.12	–
E-2-Nonenal	2.56	1.47	1.16
2-Methoxy-4-vinylphenol	3.32	0.43	–
4,7-Dihydroisobenzofuran-1,3-dione	3.52	22.02	–
3-Hydroxy-4-methoxymandelic acid	3.73	0.16	–
Apiol	4.65	0.41	13.37
3-Butylidenephthalide	4.89	20.85	–
5,7,8-Trimethyldihydrocoumarin	5.16	6.73	–
Vanillin	5.47	–	0.002
Ferulic acid	5.68	1.67	–
n-Hexadecanoic acid	5.91	8.10	0.77
β-Sesquiphellandrene	6.31	–	9.38
Elemicin	6.46	–	1.78
1,*E*-11, *Z*-13-Octadecatriene	6.47	–	3.69
3-*n*-Butylphthalide	7.06	–	0.72
Coniferol	7.53	–	0.99
Psoralen	7.98	–	2.62
Falcarinol	9.09	–	17.56
3,6,7-Trimethoxy-isobenzofuran-13(H)-one	9.23	–	4.99
Octadecanoic acid	9.69	–	0.42
Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	11.12	–	1.79
Tetracosanoic acid	12.12	–	2.74
Stigmasterol	13.64	–	11.65
β-Sitosterol	13.83	–	7.78

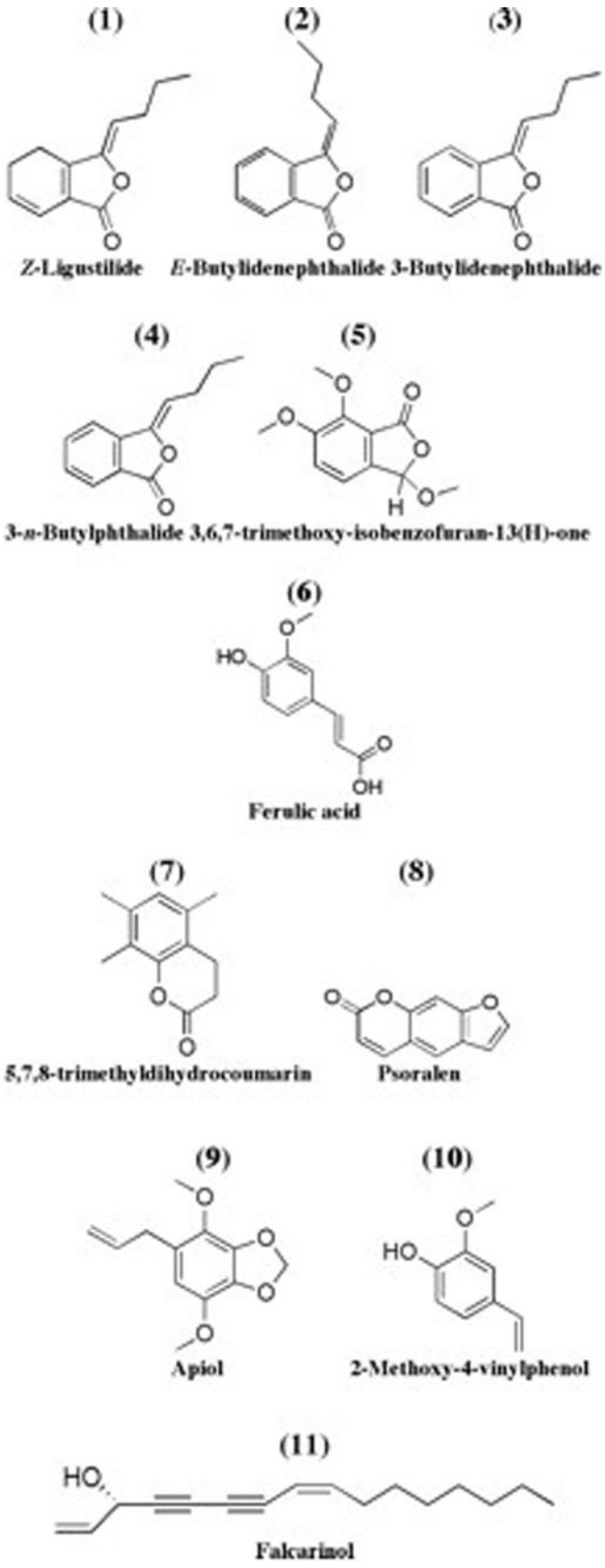

Fig. 9 Chemical structures of *Z*-Ligustilide (**1**), *E*-Butylidenephthalide (**2**), 3-Butylidenephthalide (**3**), 3-*n*-Butylphthalide (**4**), 3,6,7-Trimethoxy-isobenzofuran-13(H)-one (**5**), Ferulic acid (**6**), 5,7,8-Trimethyldihydro-coumarin (**7**), Psoralen (**8**), Apiol (**9**), 2-Methoxy-4-vinylphenol (**10**) and Falcarinol (**11**)

Table 3 Components in the aerial parts of *L. porteri* (relative abundance, %)

Fraction	Component	R.T. (min)	%
I	Carvacrol	3.68	13.79
	α-Copaene	4.24	0.93
	α-Bourbonene	4.27	0.96
	Germacrene D	4.51	19.94
	β-Bisabolene	4.58	33.80
	α-Calacorene	4.70	5.94
	Cadala-1(10),3,8-triene	5.01	2.04
	3-(4,8,12-Trimethyltridecyl) furan	5.58	22.56
II	Myristicin	7.67	12.76
	Apiol	8.27	87.23
III-A	Elemicin	4.71	32.16
	α-Spathulenol	4.80	48.88
	α-Cadinol	4.97	7.67
	6-Isopropenil-4,8a-dimethyl-1,2,3,5,6, 7,8,8a-octahydro-naphthalen-2-ol	5.06	1.83
III-B	Elemicin	4.69	1.42
	α-Spathulenol	4.82	7.73
	Apiol	4.89	57.23
	E-Butylidenephthalide	5.03	6.35
	(7R,8R)-bicyclo(5,3,1)undec-1-ene-8-ol, 7-methyl-4-(1-methyletilidene)	5.06	22.46
IV	Carvacrol	2.40	53.48
	Dihydroactinidiolide	4.11	2.38
	2,5-Octadecadiynoic acid, methyl ester	4.33	31.99
	Phytone	4.94	12.13
V	Oplopanone	8.97	1.02
VI	Scoparone	5.43	100
VII	Phytol	5.03	39.97

and *β*-hydroxypropiovanillone (2.12%) were some of the most abundant components in the analyzed samples (Table 4). Stahl-Biskup and Wichtmann (1991) mentioned that the absence of phthalides in the callus extracts of *Levisticum officinale* was due to the lack of resiniferous ducts that are necessary for their biosynthesis and a minimum degree of differentiation was necessary for this biosynthesis.

3-Butylidenephthalide was the only phthalide detected in one stem (0.82%) and two petiole callus extracts (0.99 and 0.26%). Although the calli had no physiological or structural differentiation, the phthalides biosynthesis was expressed. The results of Smitt et al. (1996) with callus, suspension cultures, elicitation of cotyledons and roots as well as genetically transformed cultures of *Thapsia garganica* revealed that no thapsigargins were present, suggesting that a cellular differentiation was needed for their biosynthesis.

3-Butylidenephthalide has acaricidal activity against larvae of *Dermatophagoides farinae* (6.77 μg $cm^{2\ -1}$) and adults of *D. pteronyssinus* (6.46 μg $cm^{2\ -1}$) (Kwon and Ahn 2002). It also has insecticidal activity against larvae of *Drosophila melanogaster* (0.94 μmol mL^{-1}) and is the most active compound compared to *Z*-ligustilide and two furanocoumarins (xanthotoxin and isompimpinellin) (Miyazawa et al. 2004). These biological activities suggest the potential of 3-butylidenephthalide as a non-toxic and biodegradable natural pesticide, at a low concentration.

Al-Abta et al. (1979) obtained 3-isobutylidene-3a,4,5,6-tetrahydrophthalide and 3-isobutylidene-3a,4-dihydrophthalide from differentiated callus with embryonary structures in the globular and hearth-shaped stages and from intact plants of *Apium graveolens* "Lathom Blanching". These cultures were grown in MS medium supplemented with 2,4-D (22.62 μM), further transferred to liquid MS medium without growth regulators, where they obtained similar quantities of these phthalides to that of the intact plant from cultures in the torpedo embryonic stage. Authors concluded that at this stage and more advanced ones, the tissues may have higher capability to accumulate or increase their

Table 4 Chemical composition in the calli extracts of *L. porteri* (relative abundance, %)

Explant	Component	R.T. (min)	%
Root I	Z-2-Heptenal	3.29	16.08
	2-Methoxy-4-vinylphenol	6.50	3.90
	n-Hexadecanoic acid	10.24	55.87
	Octadecanoic acid, methyl ester	10.88	0.82
	Hexadecanoic acid, 2,3-dihydroxypropyl ester	12.55	13.91
Seeds	Benzothiazole	5.95	0.74
	Propanoic acid, 2-methyl-, 3-hydroxy-2,4,4-trimethylpentyl ester	6.84	8.52
	1-Hexadecanol	9.72	8.00
	n-Hexadecanoic acid	10.21	45.88
	Octadecanoic acid, methyl ester	10.88	2.64
	9-Hexadecanoic acid	11.02	34.19
Cotyledon	4-Metoxyicinnamate	11.80	100
Leaf	Acetovanillone	5.40	2.65
Root II	5-Methyl-2-furancarboxaldehyde	1.78	8.57
	5-Hydroxymethyl-2-furancarboxaldehyde	3.78	85.04
	n-Hexadecanoic acid	8.06	2.10
Petiole I	2-Methoxy-4-vinylphenol	4.87	46.92
	n-Hexadecanoic acid	8.73	45.38
Stem	Carvacrol	3.21	0.06
	(*E*, *E*)-2,4-Decadienal	3.22	3.65
	2-Methoxy-4-vinylphenol	3.31	1.90
	Apiol	4.65	0.53
	3-Butylidenephthalide	4.87	0.82
	n-Hexadecanoic acid	5.88	10.65
	Falcarinol	6.15	11.56
	(11*E*, 13*Z*)-Octadeca-1,11,13-triene	6.45	9.25
	1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester	7.60	39.39
	Stigmasterol	9.70	0.39
	22,23-Dihydro-stigmasterol	9.96	1.21
Petiole II	(*E*, *E*)-2,4-Heptadienal	1.72	0.41
	2-Methoxy-4-vinylphenol	3.31	0.17
	(*E*, *E*)-2,4-Decadienal	3.34	4.82
	Apiol	4.65	21.05
	3-Butylidenephthalide	4.87	0.99
	Hexadecanal	5.00	0.29
	Eicosane	5.30	0.43
	trans-Phytol	5.44	3.50
	Tridecanoic acid, methyl ester	5.75	0.95
	n-Hexadecanoic acid	5.92	5.35
	Isopropyl palmitate	6.08	1.98
	Falcarinol	6.15	3.01
	trans-9-Hexadecen-1-ol	6.29	2.14
	Phytol	6.36	0.08
	9-Octadecynoic acid	6.48	11.43
	9,12,15-Octadecatrienal	6.50	1.17
	Octadecanoic acid	6.55	3.47
	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	7.56	6.44
	Stigmasterol	9.71	10.64
	22,23-Dihydro-stigmasterol	10.01	3.06

Table 4 continued

Explant	Component	R.T. (min)	%
Root III	(*E*, *E*)-2,4-Decadienal	3.34	5.40
	1,2-Benzenedicarboxylic acid, dihexyl ester	5.87	13.45
	1,*E*-11,*Z*-13-Octadecatriene	6.44	20.60
	1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester	7.60	3.88
	β-Sitosterol	9.96	8.41
Petiole III	(*E*, *E*)-2,4-Decadienal	3.34	0.88
	Apiol	4.65	5.60
	3-Butylidenephthalide	4.87	0.26
	trans-9-Hexadecen-1-ol	5.61	0.03
	Tridecanoic acid, methyl ester	5.75	0.27
	n-Hexadecanoic acid	5.89	11.65
	9-*endo*-Hydroxy-9-exo-vinylbicyclo(4,2,1)-nona-2,4-diene	5.97	0.61
	Falcarinol	6.15	12.91
	9,12-Octadecadienoic acid, methyl ester	6.31	0.24
	9,12-Octadecadienal	6.46	13.60
	Octadecanoic acid	6.54	0.41
	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	7.56	1.25
	Stigmasterol	9.70	4.57
	22,23-Dihydro-stigmasterol	9.98	9.33

biochemical ability to synthesize phthalides regarding the differentiation of specific cellular types.

Watts et al. (1984) obtained 3-*n*-butylphthalide and sedanolide in vitro from suspension cultures of *A. graveolens* in liquid MS medium supplemented with 2,4-D (2.26 μM) and Kinetin (2.79 μM). They observed that the substitution of 2,4-D for 3,5-dichlorophenoxyacetic acid in illuminated and aggregated cultures, the phthalides biosynthesis increased along with the terpenoid limonene.

One of the hypotheses is that the secondary metabolite biosynthesis in non-differentiated cellular cultures can be achieved with the modification of the growth pattern. Watts et al. (1985) compared the relationship among the cellular differentiation, the secondary metabolite biosynthesis and the greening of the suspension cultures of *A. graveolens.* They concluded that phthalides and limonoid biosynthesis decreased when the cellular aggregation increased and with greening.

Conclusions

Because *L. porteri* is a vulnerable plant in its natural environment and there is a huge market for its roots, the present study is very important, as it demonstrates an efficient and simple protocol that enables the potential use of its organs as an alternative source of phthalides, which will contribute to its conservation.

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