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MEDICINAL PLANTS

In vitro propagation, lactucin quantification, and antibacterial activity of Indian lettuce (*Lactuca indica* L.)

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Abstract

Lactuca indica L. (Asteraceae), a wild lettuce, is used as a vegetable and in traditional medicine. This study aims to establish *in vitro* propagation protocol and evaluate lactucin and antibacterial property from *in vitro* and natural plant tissues. Leaf blades and petioles were cultured *in vitro* on Murashige and Skoog (MS) medium supplemented with various concentrations of 6-benzylaminopurine (BAP) and 1.2 mg L^{-1} indoleacetic acid (IAA). Leaf petioles and a lower BAP concentration (0.5 mg L^{-1}) were optimal for direct shoot induction, while the leaf blade and a higher BAP (4 mg L^{-1}) concentration performed best for callus induction. When the callus was subcultured, 98.7% of samples regenerated plants on MS medium supplemented with 1.5 mg L^{-1} BAP and 0.5 mg L^{-1} IAA. MS medium containing 1 mg L^{-1} IAA was best for *in vitro* rooting. A high-performance liquid chromatography analysis of the *in vitro* samples revealed a higher amount of lactucin (sesquiterpene lactones) in the root than the callus and the leaf, whereas in naturally grown plants, higher lactucin amounts were obtained from the juvenile root followed by the root of the flowering plant and juvenile leaf as the lowest concentration. All tissue extracts showed antibacterial activity against *Pseudomonas fuscovaginae* (a rice pathogen) and *Escherichia coli*, which was directly proportional to amount of lactucin produced. This *in vitro* regeneration and phytochemical investigation will facilitate the further exploitation of this useful wild plant.

Keywords Medicinal plant · HPLC · Sesquiterpene lactone · Tissue culture · Antimicrobial activity

Introduction

Lactuca indica L., popularly known as Indian lettuce, is a lactiferous medicinal herb in the Asteraceae. It is widely distributed across Asian countries, including the Republic of Korea, Indonesia, China, Japan, India, and Malaysia (Jeffrey 1966; Oliya *et al*. 2018). The leaves of this plant have long been used in salads, soups, and fermented foods (called "Kimchi" in Korea), as well as to wrap rice or meat. Traditionally, it is used in a tonic to heal fever, coughs, diabetes, and stomach disorders. The plant has antiinflammatory, antidiarrhea, cytotoxicity, antibacterial, antidiabetic, antioxidant, α-glucosidase-inhibiting, and immune-enhancing activities (Hou *et al*. 2003; Wang *et al*. 2003; Kim *et al*. 2007, 2008, 2010; Harikrishnan *et al*. 2011; Lüthje *et al*. 2011; Park *et al*. 2014; Choi *et al*. 2016; Ha *et al*. 2017; Oliya *et al*. 2018). Like other *Lactuca* species, its leaves and stems release a milky latex upon wounding, which becomes sticky when exposed to air, helping to defend against herbivorous insects (Agrawal and Konno 2009). The latex is rich in various bioactive compounds, such as phenolics and sesquiterpene lactones (Abarca *et al*. 2019; Ilgün *et al*. 2020; Sessa *et al*. 2000). Sesquiterpene lactones are terpenoids whose structure is based on a guaiane skeleton bearing a lactone ring (Graziani *et al*. 2015). Various sesquiterpene lactones including lactucin, 8-desoyllactucin, and lactucopricin have been identified in the leaf and root of this plant (Nishimura *et al*. 1986; Michalska *et al*. 2009; Ha *et al*.

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2017). Methanol extracts of *L. indica* exhibit antibacterial, anticarcinogenic, antidiabetic, antioxidant, and anticholesterolemic activity (Hou *et al*. 2003; Wang *et al*. 2003; Ghantous *et al*. 2010; Ha *et al*. 2017). Lactucin isolated from *Lactuca species* showed analgesic, sedative, and antimalarial activity (Bischoff *et al*. 2004; Wesołowska *et al*. 2006). In addition to its medicinal and therapeutic properties, *Lactuca indica* has a strong potential for breeding as a crop in its own right or as a possible candidate for improving the traits of cultivated lettuce (*Lactuca sativa* L.) (Kim *et al*. 2012; van Treuren *et al*. 2013; Lebeda *et al*. 2014; Ha *et al*. 2017; Oliya *et al*. 2018).

Increasing demand, unregulated harvesting, and the lack of a proper strategy for replenishing medicinal plants such as *Lactuca indica* have led to a decline in these natural resources in their natural habitat (Anis and Ahmad 2016). In nature, *Lactuca indica* propagates through seed production, but as with other lettuces, the seed has low viability and dormancy problems when the light requirement for seed germination is offset by low temperature (Eenink 1977; Sakpere *et al*. 2011; Obembe *et al*. 2017). Vegetative propagation is also possible, but limited. These are the factors limiting the sustainability and large-scale cultivation of this species; however, these obstacles could be solved through the use of a tissue-culture technique, which is used for mass propagation, disease-free plant production, genetic improvement, and analyzing secondary metabolites for pharmaceutical applications (Pink 1992; Ayan and KevseroĞlu 2007; Georgiev *et al*. 2011; Al Khateeb *et al*. 2012; Salgotra and Gupta, 2015). Also, this method provides a continuous supply of potential resources without any seasonal and environmental limitations, which ensures their practical application on a commercial scale (Kumari *et al*. 2016). Most of the *in vitro* regeneration research in lettuce has focused on the cultivated lettuce (*Lactuca sativa*) (Koevaxy *et al*. 1978; Alconero 1983; Webb *et al*. 1984; Pink 1992; Teng *et al*. 1992; Ampomah-Dwamena *et al*. 1997; Park and Lim 1997; Pink and Carter 1987; Mohebodini *et al*. 2011; Latif *et al*. 2014; Ahsan *et al*. 2015; Armas *et al*. 2017), with very few focusing on wild lettuce (Koevaxy *et al*., 1978; Obembe *et al*. 2017).

Investigation of the *in vitro* cultivation of *Lactuca indica* is still lacking; therefore, the objectives of this study are (1) to develop a protocol for *in vitro* propagation via direct and indirect organogenesis using leaf-blade and leaf-petiole explants; (2) to quantify the lactucin concentrations of *in vitro*–developed leaves, callus, and roots, and compare them with the quantities found in the roots, leaves, and stems of field-grown plants at the juvenile and flowering stages using high-performance liquid chromatography (HPLC); and (3) to compare the antibacterial performance of *in vitro*–grown tissue extracts with that of field-grown plant tissues collected at the juvenile and flowering stages.

Materials and methods

In vitro propagation - Plant materials, explant preparation, media, and culture conditions The plant material used in this study was obtained from the National Gene Bank of Korea (http://www.seedbank.re.kr). The seeds were grown in a soil bed in the Crop Genomics Lab, College of Agriculture and Life Science, Seoul National University, Seoul, Republic of Korea. After 15 d, young aerial tissues (leaf blade and petiole) were collected for *in vitro* propagation. The tissues were washed under running tap water for 20 to 30 min, after which they were treated with liquid detergent (20 mL L^{-1} Tween-20 solution; Sigma-Aldrich, St. Louis, MO) for 30 min and washed five times with sterile distilled water. The pretreated tissues were then transferred into a laminar flow cabinet (Peak Scientific Instruments, Seoul, Republic of Korea) and surface-sterilized with 70% ethanol (Oci Company, Seoul, Republic of Korea) for 3 min before being treated with 1.5% sodium hypochlorite solution (*v/v*) (Oci Company, Seoul, Republic of Korea) for 8 min then washed three times (3 min per rinse) with sterile distilled water. After sterilization, the explants were left on sterile filter paper in a sterile Petri plate to absorb excess moisture. A sterile tissue-culture blade was used to prepare leaf-petiole and leaf-blade explants 0.5 cm in diameter, which were cultured in an *in vitro* culture establishment (ICE) medium.

The basal culture medium used in this study was Murashige and Skoog (MS) (Murashige and Skoog 1962) medium, containing the vitamins described by Gamborg *et al*. (1968) (Sigma-Aldrich) and 30 g L^{-1} sucrose, and with a pH of 5.8. Plant growth hormones such as cytokinins, 6-benzyl amino purine (BAP) (Sigma-Aldrich), and auxins indole acetic acid (IAA) (Sigma-Aldrich) and indolebutyric acid (IBA) (Sigma-Aldrich) were added in various concentrations, as outlined below. The pH of the media was adjusted with either 0.1 N NaOH or HCl, then supplemented with 8% agar (Sigma-Aldrich). A 50-mL aliquot of medium was poured into a tissue-culture glass bottle sealed with a transparent lid and autoclaved at 121°C for 20 min at 15 kg m^{-1} s^{-2} of pressure.

For ICE, leaf-blade and petiole explants were inoculated onto solid MS media supplemented with one of four concentrations (0.5, 1.0, 2.0, or 4.0 mg L^{-1}) of BAP with 1.2 mg L^{-1} of IAA. MS medium without growth hormones was used as the control. A sub-culture to the same hormonal combination was performed every 15 d. For the regeneration of multiple shoots, healthy calluses (green and compact but without shoot bud) developed in the ICE phase were cut to 1 cm^2 and transferred to MS media supplemented with one of four concentrations (0.5, 1.0, 1.5, or 2.0 mg L^{-1}) of BAP combined with a fixed concentration (0.5 mg L^{-1}) of IAA. *In vitro*–regenerated healthy shoots were excised and

transferred to full-strength MS medium supplemented with 1.0 or 2.0 mg L^{-1} of IAA or IBA. Each treatment was performed with ten replicates (five tissue-culture glass bottles each inoculated with two explants) and kept at 25 ± 2°C under an 8-/16-h dark/light photoperiod using cool white fluorescent light (with 4000 lux) at 23°C to 25°C during the day and 20°C at night under 60 to 70% relative humidity. For each culture, the responses were examined after 45 d of culture.

Hardening and acclimatization *In vitro*–developed plantlets (45 d old) were transferred to the acclimatization room 5 d before being transferred into the soil. The media attached to the root surface were removed from the root surface by careful washing, after which the plantlet was planted in a 500-mL plastic pot filled with a mixture of perlite, vermiculite, and soil (1:1:1). The plants were covered with a transparent polythene bag to maintain high environmental humidity and kept in the controlled room, providing the same environment in which it was cultured. The potted plants were watered with a half-strength MS basal medium devoid of sucrose at 3-d intervals for a period of 2 wk. After 7 d, the cover was removed 3 to 4 h daily to expose the plant to the air. When the plants were well adapted to the controlled room, they were transferred to the greenhouse, where the cover continued to be removed for 3 to 4 h daily. After a further month, the surviving plants were transferred into the field under natural conditions.

Lactucin quantification and antibacterial assay - *Plant tissue selection, collection, and extraction* The *in vitro*–grown leaves, calluses (green color, hard texture), and roots were collected after 45 d of culture and stored at −20°C. The same accession used for tissue culture was sown in the greenhouse, and the 3-wk-old seedlings were transplanted into the field at Seoul National University experimental farm, Suwon, Republic of Korea (N 37° 16′ 12.094″, E 126° 59′ 20.756″). The *in vivo* sample was harvested at the juvenile stage (in July) and flowering stage (in September) and separated into leaf, stem, and root tissues. All collected samples (*in vitro* and naturally grown) were freeze-dried for 48 h after harvesting and stored at 4°C until required.

The freeze-dried samples were ground to a fine powder in the grinder. The lactucin extraction was performed using the method developed by Willeman *et al*. (2014) with some modifications. In brief, 0.1 g of tissue powder was dissolved in 1.5 mL of a solvent mixture of water:chloroform:methanol (30:30:40, *v:v:v*) and mixed by vortexing after which the sample was incubated in a water bath at 60°C for 1.5 h. During the incubation period, the samples were vortexed gently every 20 min. The tubes were agitated using a shaking incubator, SI-64 (Hanyang Science Lab Co., Seoul, Republic of Korea), for 24 h in the dark at room temperature and centrifuged at 12,000 rpm at 4°C for 10 min using Micro Refrigerated Centrifuge (Smart R17, Hanil Science Industrial, Incheon, Korea). The supernatant was filter-sterilized and stored at −20°C until used in the HPLC analysis and bioassays.

Lactucin quantification using HPLC Lactucin was characterized using a Thermo Dionex ultimate 300 HPLC (Thermo Fisher Scientific, Seoul, Republic of Korea) equipped with a UV-visible diode array detector (190 to 400 nm scanning) and monitored with an Inno-C-18 column (250 mm × 4.6 mm i.d., 5 µm particles; YoungJin Biochrom, Seongnam-si, Republic of Korea). A mobile phase of buffer A (0.1% trifluoroacetic acid in distilled water) and buffer B (acetonitrile) was used in the following solution gradient: 5% buffer B at 0 min, 5% B at 1 min, 50% B at 30 min, 90% B at 31 min, 90% B at 36 min, 5% B at 37 min, and 5% B at 40 min. The flow rate was 0.8 mL min^{-1}. Thermo Dionex Chromeleon 7.2 software (Thermo Fisher Scientific, Seoul, Republic of Korea) was used to calculate the lactucin content. Lactucin (CS30062-69727; Extrasynthese, Genay, France) was used as a standard compound. All the reagents and compounds used were of HPLC grade.

Antibacterial activity The *Lactuca indica* extracts were tested for their potential antimicrobial activity in an agar disk diffusion assay using the Gram-negative bacteria *Pseudomonas fuscovaginae* and *Escherichia coli*. *P. fuscovaginae* is a soil bacterium that is pathogenic to rice (*Oryza sativa* L.), causing brown sheath rot, while *E. coli* colonizes in the intestines of mammals and can be pathogenic. The bacteria (5×10^5 $CFUmL^{-1}$) were independently inoculated into molten Mueller–Hinton agar plates. A sterile filter paper disk (diameter 6 mm) was saturated with 40 µL of the tissue extract (10 µL was dispensed at a time and allowed to dry for 10 min before the next 10 µL was dispensed) and gently pressed onto the surface of the bacteria-seeded agar plate. For each bacterial strain, a positive control (10 µL of 1 mg L^{-1} ampicillin) and a negative control (sterile disk saturated with methanol) were used. The plates were incubated overnight at 28°C for *P. fuscovaginae* and 37°C for *E. coli*. The diameter of the zone of any growth inhibition was measured on each plate, and the area was calculated and compared with the zone of growth inhibition achieved by the positive and negative controls. Three test disks were used on each Petri dish, and three replicates were performed.

Statistical analysis Each treatment for the *in vitro* study comprises two explants contained in a tissue-culture glass bottle, and all treatments were replicated five times, totaling $N = 10$ explants per treatment group. For the lactucin quantification and antibacterial assays, three replications per treatment were used. All the data were analyzed using

one-way analysis of variance (ANOVA) and the mean values for each parameter were compared for a significant difference using Duncan's multiple range test (DMRT) at $P \leq 0.05$. The results were expressed as means ± standard error for each experiment. All statistical analyses were performed using the SPSS computer software (version 23; IBM, Armonk, New York).

Results

***In vitro* culture initiation** The petiole explants were able to produce both calluses and direct shoots upon ICE. Depending on the medium used, it took 5 to 7 d to induce callus cultures and 4 to 12 d for direct shooting. Among the various concentrations of BAP used in the MS media, in combination with a constant concentration (1.2 mg L^{-1}) of IAA, 4.0 mg L^{-1} BAP was significantly more able to generate calluses on 72.0% explants of the total petiole cultured, followed by 2.0 mg L^{-1} BAP (55.0%), 1.0 mg L^{-1} BAP (25.0%), and 0.5 mg L^{-1} BAP (10.7%), respectively. By contrast, the lowest BAP concentration (0.5 mg L^{-1}) was significantly better for direct shooting, under which 87.3% of explants produced shoots; 1.0, 2.0, and 4.0 mg L^{-1} BAP induced shoots in 69.3%, 12.0%, and 0.3% of samples, respectively (Table 1, Fig. 1*A–D*). The leaf-blade explants took 9 to 12 d to induce a callus. There was no significant difference between the time taken for callus and shoot induction from the leaf-blade and leaf-petiole explants. The callus (greenish and compact) covered the leaf blade under the different media treatments after 3 wk of culture. Among the media used, 4.0 mg L^{-1} BAP induced calluses in significantly more explants (94.7%) than the other concentrations (2.0, 1.0, and 0.5 mg L^{-1} BAP induced calluses in 84.7%, 74.0%, and 72.7% of samples, respectively; Table 1, Fig. 1*E–H*).

Callus regeneration The callus regeneration began after 2 wk of culture. The medium supplemented with 1.5 mg L^{-1} BAP and 0.5 mg L^{-1} IAA regenerated significantly more callus cultures (98.7%) into shoots than the other media, producing an average of 8.5 shoots with a mean length of 4.3 cm. MS media supplemented with 1.0 or 2.0 mg L^{-1} BAP and 0.5 mg L^{-1} IAA led to the regeneration of shoots from 84% and 77% of calluses, respectively. The 1.0 mg L^{-1} BAP medium regenerated an average of 4.6 shoots per callus, with a mean shoot length of 3.8 cm, while the 0.5 mg L^{-1} BAP medium produced an average of 2.1 shoots per callus, with a mean shoot length of 2.1 cm (Table 2, Fig. 1I, J).

***In vitro* rooting, hardening off, and acclimatization** The induction of roots on the microshoots was detected 15 d after their transplant onto rooting media. Among the auxin concentrations used, all microshoots produced roots in the media supplemented with 1.0 mg L^{-1} IAA, which was followed by 99.5% of microshoots on 1.0 mg L^{-1} IBA, 96.4% of microshoots on 0.5 mg L^{-1} IAA, and 92.5% of microshoots on 0.5 mg L^{-1} IBA. There was no significant difference between the overall root induction success on 1.0 mg L^{-1} of IAA or IBA; however, 1.0 mg L^{-1} IAA induced significantly more and longer roots (an average of 7.5 roots per callus, 3.9 cm in length; Table 3, Fig. 1*K*). Similarly, 0.5 mg L^{-1} IAA and IBA performed second and third best for the percentage of root induction and root lengths, with no significant difference between them (Table 3). The *in vitro*–rooted plants acclimatized to the pots of soil in the greenhouse, displaying a 100% survival rate in both the controlled room and the greenhouse (Fig. 1*L*). These surviving plants were successfully acclimatized in the field.

Lactucin quantification using a HPLC analysis Using a HPLC analysis, lactucin (standard 1-Lactucin-17.087) was detected

Table 1 The effect of plant growth regulators on callus and direct shoot bud induction from *Lactuca indica* L. leaf explants 45 d after inoculation for *in vitro* culture

Explants	Plant growth regulators (mg L^{-1}) in MS medium		Callus induction (%)	Days to callus induction	Direct shoot induction (%)	Days to shoot induction
	BAP	IAA				
Leaf petiole	0.5	1.2	10.7 ± 2.9^{d}	5.8 ± 0.9	87.3 ± 1.5^{a}	4.9 ± 0.4^{a}
	1.0	1.2	25.0 ± 0.7^{c}	7.0 ± 1.0	69.3 ± 1.7^{b}	6.8 ± 0.9^{b}
	2.0	1.2	55.0 ± 2.9^{b}	6.2 ± 0.9	12.0 ± 1.2^{c}	12.7 ± 0.6^{c}
	4.0	1.2	72.0 ± 1.5^{a}	5.5 ± 0.8	5.3 ± 0.9^{d}	11.7 ± 0.7^{c}
Leaf blade	0.5	1.2	72.67 ± 1.5^{c}	11.7 ± 0.6	-	-
	1.0	1.2	74.00 ± 3.8^{c}	9.7 ± 1.2	-	-
	2.0	1.2	84.67 ± 0.9^{b}	11.0 ± 1.2	-	-
	4.0	1.2	94.67 ± 0.9^{a}	11.7 ± 2.7	-	-

Note: *MS* Murashige and Skoog (Murashige and Skoog 1962) medium; *BAP*: 6-benzyl amino purine; *IAA* indole acetic acid. Data are presented as means ± standard error. Values followed by different *superscript letters* (*a*, *b*, *c*, *d*) in the same *column* are significantly different ($P < 0.05$, Duncan's multiple range test)

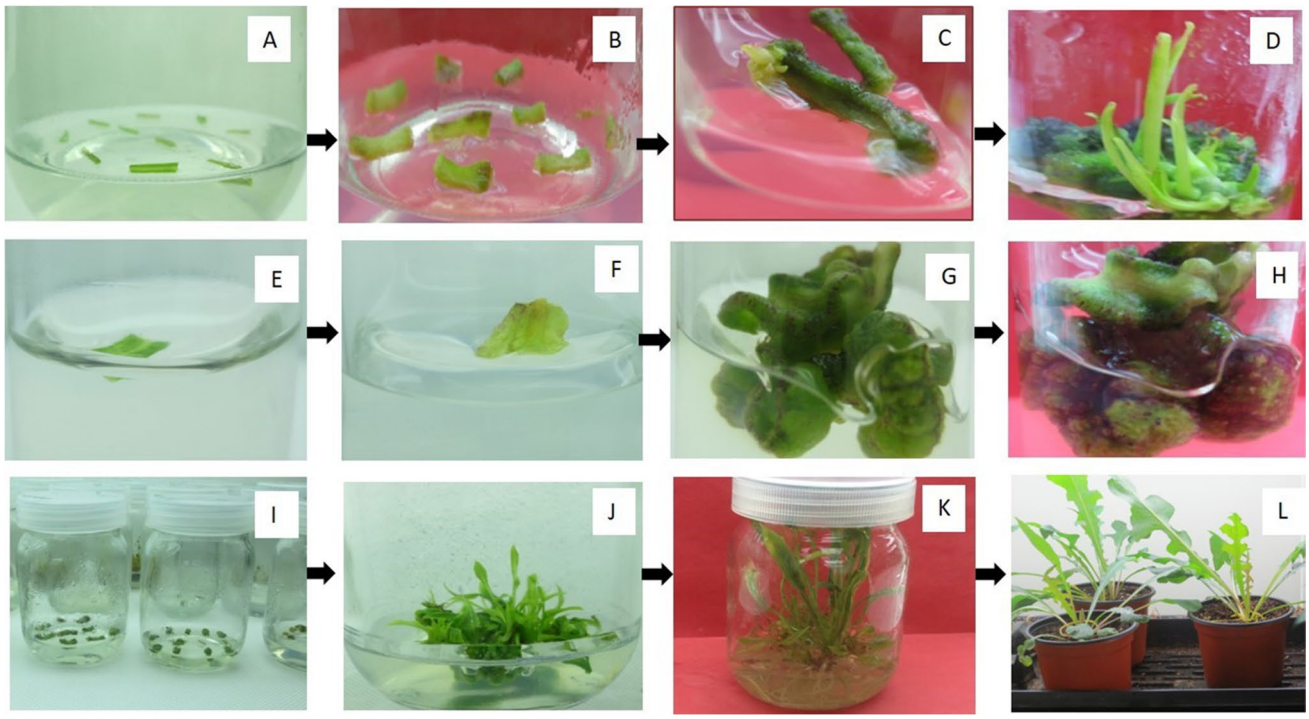

Figure 1. *In vitro* shoot regeneration of *Lactuca indica* L. (*A–D*) Petiole explant response on Murashige and Skoog (MS) medium containing 0.5 mg L^{-1} 6-benzyl amino purine (BAP) and 1.2 mg L^{-1} indole acetic acid (IAA) at different stages of development: (*A*) petiole explant at the time of inoculation; (*B*) petiole enlargement after 5 d; (*C*) induction of shoot buds at 16 d; and (*D*) elongation of shoots at 22 d. (*E–H*) Leaf explant response on MS medium containing 4.0 mg L^{-1} BAP and 1.2 mg L^{-1} IAA: (*E*) leaf explant at the time of inoculation; (*F*) leaf enlargement after 5 d; (*G*) callus induction after 16 d; and (*H*) callus hardening after 22 d. (*I–L*) Callus culture on MS medium containing 1.5 mg L^{-1} BAP and 0.5 mg L^{-1} IAA: (*I*) callus inoculated in the media; (*J*) shoot regeneration after 30 d; (*K*) *in vitro*–rooted plant on MS medium containing 1.0 mg L^{-1} IAA after 30 d of culture; (*L*) well-adapted plant in greenhouse after four wk of acclimatization.

Table 2 The effect of different concentrations of BAP with 0.5 mg L^{-1} IAA for shoot induction and elongation of *Lactuca indica* L. from callus cultures. Number and length of shoots after 45 d of culture

Plant growth regulators (mg L^{-1}) on MS medium		Percentage of calluses producing multiple shoots (%)	Number of shoots per explant	Shoot length (cm)
BAP	IAA			
0.5	0.5	77.0 ± 0.6^{c}	2.1 ± 0.2^{c}	2.1 ± 0.2^{d}
1.0	0.5	84.3 ± 0.4^{b}	4.6 ± 0.3^{b}	3.8 ± 0.2^{b}
1.5	0.5	98.7 ± 0.7^{a}	8.5 ± 0.4^{a}	4.3 ± 0.2^{a}
2.0	0.5	86.7 ± 1.9^{b}	3.8 ± 0.2^{b}	3.2 ± 0.1^{c}

Note: *MS* Murashige and Skoog (Murashige and Skoog 1962) medium; *BAP* 6-benzyl amino purine; *IAA* indole acetic acid. Data are presented as means ± standard error. Values followed by different *superscript letters* (*a*, *b*, *c*, *d*) in the same *column* are significantly different ($P > 0.05$, Duncan's multiple range test)

at a retention time of 17 min in all tissue extracts. Its concentration ranged from 0.2 to 2.2 µg g^{-1}, with an average of 0.9 µg g^{-1}. Among the *in vitro* samples, the highest concentration of lactucin was detected in the root (0.9 µg g^{-1}) followed by the callus (0.7 µg g^{-1}) and the leaf (0.2 µg g^{-1}). In the naturally grown plants, the highest concentration of lactucin was detected in the root at the juvenile stage (2.2 µg g^{-1}), followed by the root during flowering (1.3 µg g^{-1}), the stem during flowering (0.8 µg g^{-1}), the stem at the juvenile stage (0.7 µg g^{-1}), the leaf during flowering (0.3 µg g^{-1}), and the leaf at the juvenile stage (0.2 µg g^{-1}) (Fig. 2*A*, *B*). The DMRT indicated that the lactucin content in the juvenile root was significantly higher than in other samples. There was no significant difference between the lactucin amounts obtained from the tissue-cultured roots, calluses, and stems, as well as the flowering stems of the naturally grown plants. Similarly, the lactucin contents of the tissue-cultured leaves and the naturally grown leaves of the juvenile and flowering

Table 3 The effect of different concentration of auxins on the *in vitro* root induction and elongation of *Lactuca indica* L. plantlets after 45 d of culture

Auxin used	Concentrations (mg L^{-1}) in MS medium	Percentage of response (%)	Number of roots/ shoots	Root length (cm)
IAA	1.0	100.0 ± 0.0^{a}	7.5 ± 0.4^{a}	3.9 ± 0.1^{a}
	0.5	96.4 ± 1.0^{b}	4.3 ± 0.3^{c}	2.7 ± 0.1^{c}
IBA	1.0	99.5 ± 0.3^{a}	6.3 ± 0.4^{b}	3.5 ± 0.1^{b}
	0.5	92.5 ± 1.5^{c}	3.9 ± 0.2^{c}	2.3 ± 0.1^{d}

Note: *MS* Murashige and Skoog (Murashige and Skoog 1962) medium; *BAP* 6-benzyl amino purine; *IAA* indole acetic acid; *IBA* indole butyric acid. Data are presented as means ± standard error. Values followed by different *superscript letters* (*a*, *b*, *c*, *d*) in the same *column* are significantly different ($P > 0.05$, Duncan's multiple range test)

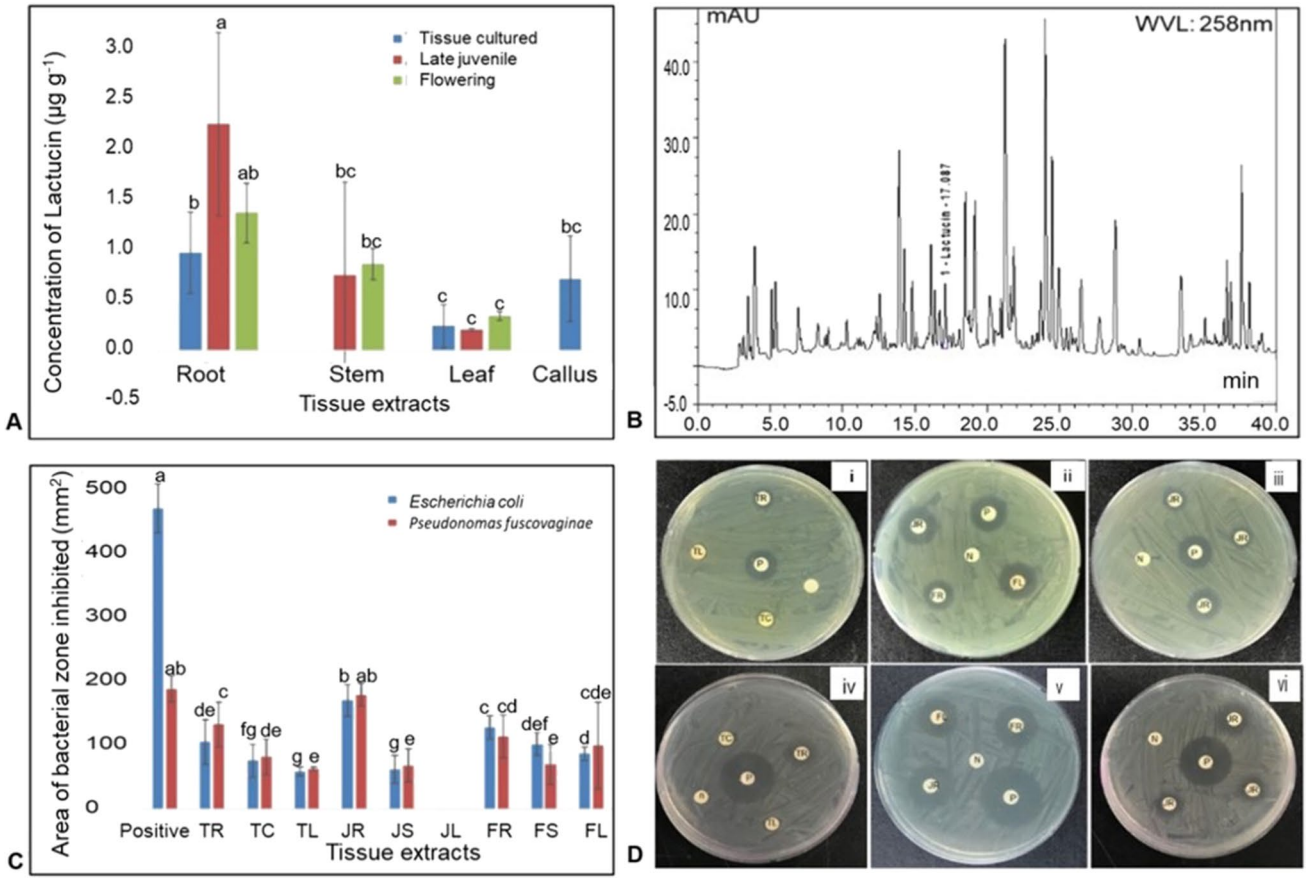

Figure 2. Characterization of lactucin and its antibacterial application. (*A*) Lactucin concentration in different *Lactuca indica* L. extracts of *in vitro*–cultured and naturally grown tissues. (*B*) High-performance liquid chromatogram indicating the detection of lactucin. (*C*, *D*) Area of the bacterial zone of inhibition (mm^2) against *P. fuscovaginae* (i–iii) and *E. coli* (iv–vi) by extracts of the various tissue types. TR: tissue-cultured root; TC: tissue-cultured callus; TL: tissue-cultured leaf; JR: root of juvenile plant; JL: leaf of juvenile plant; JS: stem of juvenile plant; FR: root of flowering plant; FS: stem of flowering plant; FL: leaf of flowering plant; N: negative control (solvent mixture: water/chloroform/methanol); P: positive control (ampicillin). The data in the *bar graphs* are presented as means ± standard error. Different *letters* indicate significant differences, as determined using Duncan's multiple range test ($P \leq 0.05$).

plants were significantly lower than those detected in the other tissues (Fig. 2A).

Antibacterial activity The tissue extracts of all plant samples (except the leaves from the naturally grown juvenile parental plants) were effective against both bacteria tested (Fig. 2C, D). The solvent mixture used as the negative control did not inhibit bacterial growth. The positive control, ampicillin, showed a larger bacterial inhibition area than test samples (465.6 mm^2) for *E. coli*; however, the area of inhibition of *P. fuscovaginae* achieved using the positive control (185.7 mm^2) was not significantly different from the juvenile root

extract (176.5 mm^2). Among the tissue-cultured samples, the root was able to kill both bacteria, achieving areas of bacterial inhibition for *E. coli* and *P. fuscovaginae* of 103.8 mm^2 and 131.0 mm^2, respectively. Using the callus extract, bacterial inhibition areas of 74.5 mm^2 and 80.7 mm^2 were achieved against *E. coli* and *P. fuscovaginae,* respectively. The leaf extract was less effective at inhibiting these bacteria where bacterial inhibition areas against *E. coli* and *P. fuscovaginae* were 47.5 mm^2 and 49.1 mm^2, respectively (Fig. 2C, D).

Among the naturally grown samples, the juvenile root was able to kill both bacteria. The areas of *E. coli* inhibition achieved (in decreasing order) by the root of the juvenile plant; the root, stem, and leaf of the flowering plant; and the stem of the juvenile plant were 168.0, 125.9, 100.3, 85.8, and 61.0 mm^2, respectively. These values were significantly different, as determined using the DMRT. For *P. fuscovaginae,* the inhibition areas obtained (in decreasing order) were 176.7, 112.1, 69.0, and 67.4 mm^2 for the root of the juvenile plant, and the root, stem, and leaf of the flowering plant, respectively. We did not identify any significant difference in the antibacterial activity of the juvenile and mature stem extracts for *P. fuscovaginae*; however, there was a significant difference in the antibacterial activities of the juvenile and mature root extract (Fig. 2*C, D*).

The *E. coli* inhibition levels achieved by the cultured tissue samples were less effective than the naturally grown juvenile and mature roots, but more effective than the stem extracts from the juvenile and flowering plants. The antibacterial activity of the callus was higher than the juvenile stem and lower than the other extracts. The tissue-cultured leaf extract was not significantly different from the juvenile stem, but significantly less effective than other extracts. Similarly, for *P. fuscovaginae*, the antibacterial activity of the tissue-cultured root was markedly lower than the juvenile root but considerably higher than the other extracts. The callus showed significantly lower antibacterial activity than the juvenile and mature root, but substantially higher activity than the other extracts. The antibacterial activity of the tissue-cultured leaf was lower than the root and leaf of the juvenile and flowering plants, but not significantly different to the stems of plants at either developmental stage (Fig. 2*C*).

Discussion

The *in vitro* propagation of a traditional medicinal plant such as *Lactuca indica* is essential for enabling various technological breakthroughs that will enhance the breeding, conservation, and research of this species (Anis and Ahmad 2016; Chavan *et al*. 2018; Oliya *et al*. 2021). Here, a protocol was developed for direct and indirect organogenesis using leaf and petiole explants. Three major factors can influence the regeneration progress: the selection of a cultivar with adequate regeneration efficiency, the optimization of the explant source material, and the adaptation of the medium (Ampomah-Dwamena *et al*. 1997). The rapid production of microshoots could be helpful for the production of pathogen-free clones, which can be used more efficiently for producing true-to-type plants in a short timeframe. In indirect organogenesis, the plant cells multiply in a disorganized manner to produce a callus, which can in turn regenerate multiple shoots, roots, and even full embryos with the proper supplementation of growth hormones, pH, and light. This indirect organogenesis is an essential prerequisite to new cultivar production, cryopreservation, genetic transformation, and large-scale high-value secondary metabolite production (Rout *et al*. 2006; Dunwell 2008).

Different tissues may contain different levels of endogenous hormones; therefore, the source material of the explants has a critical impact on callus induction and regeneration success (Das *et al*. 2013; Oliya *et al*. 2021). Different studies in lettuce tissue culture have focused on ranking different cultivars according to their callus formation or direct shoot induction capacities (Ampomah-Dwamena *et al*. 1997; Latif *et al*. 2014). Leaves and leaf petioles have been used to generate explants in several plants to facilitate direct and indirect embryogenesis (Soneji *et al*. 2002; Arumugam *et al*. 2009; Ahsan *et al*.2015). These reports showed that genotype, explant age, plant growth hormone concentrations, the amount of micro- and macronutrients, and the carbon source all play a vital role in regeneration. Callus and shoot induction using various explants have been performed in wild and cultivated lettuce (Park and Lim 1997; Mohebodini *et al*. 2011; Ahsan *et al*. 2015; Armas *et al*. 2017; Obembe *et al*. 2017) however, our study is the first to achieve the *in vitro* propagation of *Lactuca indica*.

A lower BAP concentration significantly increased the shoot induction rate, while a higher concentration of BAP increased the callus induction rate, which is consistent with previous findings in cultivated lettuce and chicory (*Cichorium intybus* L.) (Park and Lim 1997; Mohebodini *et al*. 2011; Armas *et al*. 2017). The MS medium supplemented with 1.5 mg L^{-1} BAP and 0.5 mg L^{-1} IAA performed best for callus regeneration, consistent with an earlier report in *Cichorium pumilum* Jacq. (Al Khateeb *et al*. 2012). Similarly, 2.5 mg L^{-1} BAP combined with 0.5 mg L^{-1} NAA performed best for shoot induction in *Launaea taraxacifolia* (Willd.) Amin ex C. Jeffrey, an African wild lettuce, using single-node culture (Obembe *et al*. 2017). Ahsan *et al*. (2015) used leaf segments of lettuce (*Lactuca sativa*) variety (BARI Lettuce-1) for *in vitro* regeneration, revealing that MS medium supplemented with 1.5 mg L^{-1} BAP and 0.1 mg L^{-1} NAA was best for callus induction, while MS medium containing 2.5 mg

L^{-1} BAP and 0.1 mg L^{-1} NAA was best for shoot induction. Callus quality and carbohydrate source were most critical for cell growth and differentiation in a suspension culture of lettuce (Teng *et al*. 1992). The highest frequency and speed of multiple shoot regeneration from cotyledon explants of *L. sativa* were recorded on MS regeneration medium supplemented with 0.5 mg L^{-1} activated charcoal, 3% sucrose, 0.5 mg L^{-1} BAP, and 0.5 mg L^{-1} NAA, which induced shoots through direct regeneration. In the present study, 1.0 mg L^{-1} IAA performed best for rooting over the other treatments, consistent with earlier studies in lettuce and chicory (Pink and Carter 1987; Yucesan *et al*. 2007; Dolinski and Olek 2013).

One of the challenges in plant tissue culture is the process of acclimatization. Under *in vitro* conditions, plants are raised in a controlled environment and with high humidity. These plants therefore have adequate moisture in their leaves and stems, resulting in their production of leaves containing large spaces between their palisade cells and few stomata. When the plant is exposed to the outer atmosphere, there is a high degree of transpiration and fungal contamination, leading to plant death. The success of acclimatization depends on the genotype, root growth, season, and handling. The periodic removal of the plastic cover to gradually lower the high atmospheric humidity, irrigation with half-strength MS basal medium devoid of sucrose for 3 wk at 3-d intervals, and raising plants in a controlled room for another 6 wk before transferring into the greenhouse or field often resulted in a 100% survival rate for *Lactuca indica* in our study. Previously, Conner *et al*. (2019) studied the *ex vivo* growth response of *Cichorium intybus* (cultivar 'Grasslands Puna') and *Lactuca sativa* (cultivar 'Cobham Green') plants regenerated from the nodal segment that had gone phage change to *in vitro* flowering (adult plant), and the cauline leaf culture of the adult plant (rejuvenated plants). The resulting rejuvenated plants for both species exhibit substantially improved performance in greenhouse conditions with increased frequency of plant survival, a doubling of the frequency of plants that flowered, and substantially increased seed production compared to adult plants. Similarly, De Souza *et al*. (2007) obtained 100% survival for *in vitro* raised plantlets of *Lychnophora pinaster*, a threatened endemic medicinal plant belonging to Asteraceae when planted in soil from the area of occurrence of the species, whereas 0% survived when planted in commercial substratum Plantmax.

In this study, the lactucin concentrations of the *in vitro*–grown roots and calluses were higher than the leaves, lower than the roots, and not significantly different from the stems of the naturally grown mother plant. This result suggests that tissue-cultured materials are similarly biochemically potent to the naturally grown plants. In general, we found roots were the best tissue for the accumulation of lactucin; however, this compound can be extracted from the entire plant. The variation in the concentration of lactucin among the tissues may be due to transcriptomic difference between the tissue, and abiotic environmental factors (Badri *et al*. 2010; Sampaio *et al*. 2016; Hubbard *et al*. 2017; Soorni *et al*. 2021). Previously, Michalska *et al*. (2009) used ultraviolet–HPLC and thin-layer chromatography to identify six different types of sesquiterpene lactones (8-deoxylactucin, jacquinelin, crepidiaside B, lactucopicrin, glucozaluzanin C, and lactuside-A) in the roots and leaves of *Lactuca indica*; however, they did not detect lactucin in this plant. In another previous study, a small amount of lactucin, 8-desoyllactucin, and lactucopricin was detected in the leaves at the bolting stage, while lactucin was only observed in the flowering stage (Ha *et al*. 2017). Moreover, in the same study, lactucin concentrations ranging from 1.9 to 98.7 μg g^{-1} were reported in the leaves of flowering plants from 61 *Lactuca indica* accessions collected across the Republic of Korea. In the present study, using a HPLC analysis, we have quantified lactucin from the leaf, stem, and root in the flowering and juvenile periods of naturally grown plants. A higher amount of lactucin was obtained from the roots of plants in the juvenile period, followed by the leaves of flowering individuals. Comparing the work of Ha *et al*. (2017) with the present study, we conclude that lactucin biosynthesis in *Lactuca indica* depends on the plant tissue involved, phenological development, and the environment.

Sesquiterpene lactones, a diverse group of terpenoids isolated from Asteraceae species, exhibit a broad spectrum of biological activities. Several of them are already commercially available as a drug, such as artemisinin isolated from *Artemisia annua* is used as an antimalarial drug (Liu *et al*. 2019; Zhang *et al*. 2019; Moujir *et al*. 2020). Here, we explored the antibacterial activity of the different tissue extracts against *P. fuscovaginae* and *E. coli*, with both bacteria reacting similarly. The root extract, which contained a significantly higher lactucin concentration, also showed a significantly higher antibacterial activity. The leaf extract, which contained a low lactucin concentration, showed weaker or no antibacterial activity. Thus, the antibacterial activity of the tissue extracts could be attributed to the presence of lactucin; however, as reported by previous studies, diverse sesquiterpenoids, phenolic, flavonoids, and minerals are also present in the crude extract and could potentially have an antibacterial effect (Nishimura *et al*. 1986; Kim *et al*. 2008; Michalska *et al*. 2009; Kim and Yoon 2014; Padilla-Gonzalez *et al*. 2016; Ha *et al*. 2017; Abdalla *et al*. 2021). A previous study by Pavlović *et al*. (2011) showed that the extracts of *Lactuca sativa* grown in greenhouses showed high antibacterial activity against various bacterial strains, particularly against *Staphylococcus aureus* and *Proteus mirabilis*.

Lactuca indica extracts have been shown to have various therapeutic activities, including antibacterial activity

against uropathogenic *E. coli* (Hou *et al*. 2003; Wang *et al*. 2003; Kim *et al*. 2007, 2010; Lüthje *et al*. 2011; Park *et al*. 2014). Lactucin in *Cichorium intybus* showed antibacterial activity against various bacteria (Petrovic *et al*. 2004; Nandagopal and Kumari 2007; Liu *et al*. 2013). Together with its derivatives lactucopicrin and 11beta,13-dihydrolactucin, which are characteristic bitter sesquiterpene lactones of *Lactuca virosa* and *Cichorium intybus*, isolated from root and leaves, lactucin was evaluated for analgesic and sedative properties in mice (Wesołowska, *et al*. 2006). Lactucin and lactucopicrin also showed antimalarial activity (Bischoff *et al*. 2004). Though the therapeutic activities of plant extracts were reported, the present study is the first to identify the antibacterial effect of *Lactuca indica* against the plant pathogen *P. fuscovaginae*. This finding broadens the therapeutic horizon of *Lactuca indica* and gives insight into its potential applicability as a pesticide. Further research on bioassay-guided fractionation and purification is strongly recommended in this field.

Conclusion

This study established an efficient *in vitro* propagation protocol for direct and indirect organogenesis, which have many potential applications for medicinal and horticultural species such as *Lactuca indica*, including the true-to-type large-scale propagation of superior genotypes and genotypic improvement via mutagenesis and/or genetic engineering. This study validates the practical application of a newly established tissue-culture protocol for phytochemical and therapeutic research. In addition, this study found variation in the lactucin concentrations and antibacterial activities, which were affected by the developmental stage of the plant, the environment, and the tissue type sampled, which is beneficial for germplasm conservation and the economic exploitation of *Lactuca indica*. Our results could guide the further study of lettuce and other medicinal plants.

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Declarations

Conflict of interest The authors declare no competing interests.

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