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In vitro propagation of *Stephania pierrei* diels and exploration of its potential as sustainable phytochemical production from tuber and callus

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Abstract

Background The tuber of *Stephania pierrei* Diels has been used for treating cardiovascular disease, migraine, and body edema and may exhibit antimalarial, anticancer, and anticholinesterase properties. It is also a popular ornamental plant. Consequently, plant tubers have been extensively harvested from the wild, posing a high risk of extinction. We assessed the in vitro propagation and essential phytochemical production from the calli of *S. pierrei*.

Results The highest callus weight (1.52–1.59 g) induced from the tuber flesh without peel occurred when using Murashige and Skoog (MS) medium with basal salts supplemented with 3–4 mg/L benzyladenine (BA) and 0.1 or 0.5 mg/L naphthaleneacetic acid. MS medium with a concentration of 3 mg/L BA was the most efficient medium for shoot regeneration, producing an average of 7.40 ± 1.140 shoots. Similarly, MS medium with 1 mg/L meta-topolin (mT) was most efficient for multiple shoot production (with an average of 13.40 ± 2.30 shoots). Root induction was successfully performed using the ½ MS medium. HPLC analysis revealed that calluses, tubers without peel, tubers with peels, and peels contained fangchinoline, cepharantine, and tetrandrine. Peels had the highest amounts of the first two alkaloids and a high amount of tetrandrine, which was related to the relative expression levels of three genes involved in the isoquinoline alkaloid biosynthesis pathway: coclaurine N-methyltransferase (*CNMT*), norcoclaurine synthase (*NCS*), and 6-O-methyltransferase (*6OMT*). GC–MS was employed for phytochemical identification of callus and tuber with peel, which revealed that out of the 24 phytochemicals identified, 13 were only found in callus and 5 were present only in tubers. DPPH scavenging percentage, ferric reducing antioxidant power, and ABTS radical cation scavenging activity assays revealed that the extracts from the four tissues showed antioxidant activities. The peel showed significantly higher total flavonoid and phenolic content, whereas the callus displayed the highest total alkaloid concentration.

Conclusions Producing secondary metabolites such as cepharantine, tetrandrine, and fangchinoline by culturing callus holds potential as a low-cost and sustainable method for producing pharmaceutical phytochemicals.

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Keywords Callus induction, Multiple shoots, Sustainable production, Phytochemical, Cepharantine, Tetrandrine, Fangchinoline, HPLC, GC–MS, Gene expression, Antioxidant

Background

Plant secondary metabolites possess an inherent capacity to generate molecules that play an essential role in ecological interactions and offer significant utility in various industries, including agriculture, cosmetics, and pharmaceuticals [1]. These metabolites include alkaloids, flavonoids, phenolics, terpenes, and steroids, which exhibit several medicinal properties, including antibacterial, antiviral, antimalarial, anticancer, antidiabetic, hepatoprotective, antiulcer, anti-inflammatory, and antimicrobial properties [2–4]. Produced by plants as defense mechanisms against pathogens and herbivores, they have been used by humans to cure several infections, illnesses, and ailments [5].

The genus *Stephania* belongs to the family Menispermaceae and comprises 60 species. It is widely distributed in the tropical and subtropical parts of Asia, Africa, and Oceania. The species of this genus are exceptional sources of several bioactive compounds with significant pharmaceutical value [6]. These plants have been utilized in traditional medicine to address various ailments, including allergies, asthma, tuberculosis, diarrhea, high blood sugar levels, cancer, malaria, and fever [7, 8]. Notably, *S. pierrei* Diels is predominantly found on the Indo-Chinese Peninsula [9, 10]. In Cambodia, the tuber of this plant has been traditionally utilized for treating cardiovascular disease, migraine, and body edema [11]. Its tuber extracts exhibit antimalarial, anticancer, and anticholinesterase properties [7, 8, 12]. Furthermore, it also serves as a constituent. Additionally, there is documented evidence regarding the use of the plant extract in Thai traditional medicine to induce skeletal muscle relaxation and provide pain relief. Our preliminary consultations with local residents of Thailand have revealed that crushed leaves of *S. pierrei* Diels have several applications, including the formation of gelatin for wound compression, hemostatic and healing support, and as an ingredient in a vegetable dish combined with chili paste (Fig. 1D). In addition, villagers in Kampong Speu Province, Cambodia, also prepare a local jelly dessert using the leaves of this plant species [13]. Beyond its pharmaceutical and culinary applications, this plant is highly valued for its ornamental appeal, particularly due to the aesthetic qualities of its tubers and leaves (Fig. 1C). The product is readily available for purchase in online and offline markets, catering to both local and foreign consumers. *S. pierrei* Diels were synonymous with *S. erecta*. Bua kua, Koh Hua Bua, and Bua bok are components of vernacular [9]. Tubers of *S. pierrei* Diels are extensively harvested from the wild (Fig. 1A, B) because of their many advantageous properties. Due to over-collection and threats from habitat destruction, the population of *S. pierrei* Diels has significantly declined, raising serious concerns about the species' sustainability. Consequently, *S. pierrei* has been proposed for classification as 'Endangered' under the IUCN criteria [13, 14]. Natural propagation of *S. pierrei* Diels involves seed dispersal; however, germination of the horseshoe-shaped endocarp is challenging.

Plant tissue culture is a well-known method for the propagation and production of secondary metabolites. It can effectively manage bacterial and heavy metal contamination. Moreover, it is not constrained by seasonal variations and can be used throughout the year owing to its ability to regulate different factors, including light, humidity, pressure, and herbivory by insects [15–18]. The tissue culture of *Stephania* is used to propagate and cultivate superior-quality seedlings. Tissue culture techniques have been used for several species of *Stephania*, including *S. epigaea*, *S. tetrandra*, *S. glabra*, and *S. cepharantha* [19–21].

As callus exhibits the capacity to generate secondary metabolites, an established in vitro callus induction system can be employed to produce substances with pharmaceutical value [22]. Callus cultures have been successfully established for various medicinal herbs, including *Phyllanthus amarus* [23], *Woodfordia fruticose* [24], *Plumbago zeylanica* [25], *Atropa acuminata* [26], and *Rhodiola imbricata* [27]. Gorpenchenko et al. [28] discovered that attempting alkaloid biosynthesis from *S. glabra* cell cultures does not work because the cells are toxic; however, certain cell types, such as vascular tissues, laticifers, and parenchymal cells with inclusions, could efficiently tolerate the alkaloid buildup. *S. venosa* callus and cell suspension cultures can produce significant quantities of dicentrine, a valuable alkaloid, indicating that such cultures can work as substitutes for producing secondary metabolites [29]. Several previous studies have used different parts of various plants (including stems, leaves, roots, and tubers) to establish successful cultures using callus for producing different secondary metabolites, such as dicentrine and stepharine [29–32].

Owing to the high extinction risk of *S. pierrei* Diels, it is imperative to investigate and identify propagation protocols for this species. The present study aimed to establish an in vitro propagation protocol for *S. pierrei*. To explore the potential of certain valuable secondary metabolites produced from calli and plant parts, as well as to determine the expression of certain genes in the isoquinoline alkaloid pathway.

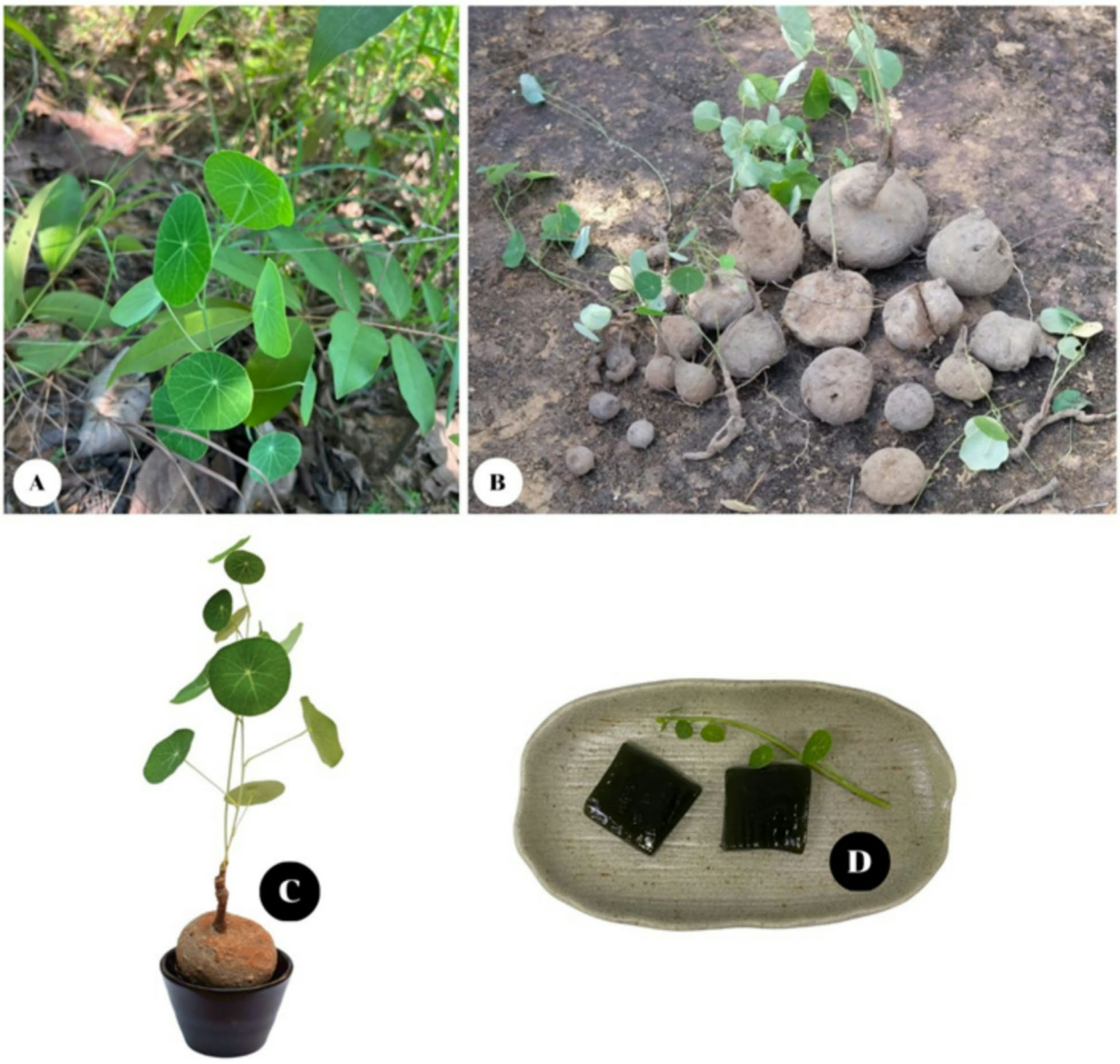

Fig. 1 Germination of *Stephania pierrei* Diels from the seed in its natural habitat at the Burapha University Sakaeo campus (**A**); *S. pierrei* Diels tubers were collected for the present study (**B**); Potted *Stephania* plants are often used for ornamental purposes (**C**); and gelatin extraction obtained by crushing the leaves of *S. pierrei*. (**D**)

Materials

Plant material preparation

In the present study, we collected specimens, the whole plant, of *S. pierrei* Diels. from a forest located on the Sakaeo campus of Burapha University, Thailand (13°44'16.9"N 102°17'11.0"E) in March 2021. Dr. Chakkrapong Rattamanee, the botanist from Burapha University's Sakaeo Campus's Faculty of Agricultural Technology, identified the plant. Healthy and strong tubers were carefully removed from the soil surface. Voucher specimens (with the identification number, Agritech-001) were stored in the herbarium of the Faculty of Agricultural Technology, Burapha University Sakaeo Campus.

The shoot, leaf, node, and tuber samples were rinsed for 10 min using tap water, and the peels of tuber samples were removed. Subsequently, a 1-minute surface sterilization was performed for all samples using 70% ethanol, following which all the samples were submerged in a solution containing 0.6% sodium hypochlorite and one drop of Tween 20 for 15 min. Although *S. pierrei* Diels is tuberous and grows close to the soil surface, hazardous chemicals, such as mercuric chloride, are not necessary for disinfection. This is because the leaf surface and node

sections were hairless, and the tuber was peeled off prior to its introduction into the tissue culture.

The samples were, then, subjected to a triple washing process using autoclaved deionized water. The explants were first transferred to growth regulator-free MS [33] medium containing 30 g/L sucrose at pH 5.7 and then solidified using 7.5 g/L agar powder. The cultures were kept under cool-white fluorescent tubes with a light intensity of 30 µmol/m^2/s and a temperature of 25 ± 2°C for at least one week until it was confirmed that there were no instances of contamination.

Callus induction

The uncontaminated samples were transferred to MS medium supplemented with 22 different combinations of plant growth regulators (PGRs) at different concentrations, namely benzyladenine (BA; 1–4 mg/L), naphthaleneacetic acid (NAA; 0.1 and 0.5 mg/L), thidiazuron (TDZ; 0.2–0.6 mg/L), and 2,4-D (1–4 mg/L) (Table 1). The cultures were kept at a temperature of 25 ± 2 °C and subjected to a photoperiod of 16 h light and 8 h dark. The experiment was performed in triplicates, and each replicate comprised 25 explants. After 45 days of in vitro culturing, we investigated the frequency of callus induction, including the callus induction rate (%) and callus weight.

Shoot induction from callus and multiple shoot initiation

The callus samples, which were about 300 mg each, were placed on an MS medium supplemented with an assortment of five PGRs at different concentration ranges: BA (1–5 mg/L), TDZ (0.2–0.8 mg/L), kinetin (1.0–3.0 mg/L), NAA (0.1–0.2 mg/L), and meta-topolin (mT); 0.5–2.0 mg/L) (Table 2). Following inoculation, the cultures were kept at a temperature of 25 ± 2°C, with a photoperiod of 16 h light and 8 h dark and photosynthetic photon flux density of 30 µmol/m^2/s under cool white fluorescent lamps for 45 days to facilitate regeneration. For multiple shoot induction, a single regenerated shoot was transferred to an MS growth regulator-free medium and cultured under a 16/8 h light regime for 30 days. Subsequently, the explants were transferred to the same media formulation used for plant regeneration from callus. The culture conditions were identical to those used in the callus induction experiments. After 45 d of culturing, data regarding the average number of shoots and their corresponding lengths were recorded in centimeters. Ten replicates were used for each experiment, and each replicate consisted of ten explants.

Root induction

We randomly selected 100 shoots derived from the calli and multiple shoots for root induction. This was done

Table 1 Effects of growth media on callus induction, callus weight, and appearance of the callus induced from three different explants of *Stephania pierrei* diels in vitro culture

No.	PGRs (mg/L)				Weight of callus (g)			Appearance of callus
	BA	NAA	TDZ	2,4-D	Shoot	Node	Tuber	
1	0	0	0		0^{h}	0^{k}	0^{e}	-
2	1	-	-	-	0^{h}	0^{k}	0.20 ± 0.50^{de}	greenish compact
3	2	-	-	-	0.82 ± 0.08^{e}	0.62 ± 0.01^{h}	1.23 ± 0.21^{b}	greenish compact
4	3	-	-	-	0.92 ± 0.03^{d}	1.05 ± 003^{e}	1.56 ± 0.03^{a}	brown compact
5	4	-	-	-	1.06 ± 0.07^{c}	1.42 ± 0.06^{b}	1.58 ± 0.16^{a}	brown compact
6	-	0.1	-	-	0^{h}	0^{k}	0^{e}	-
7	-	0.5	-	-	0^{h}	0^{k}	0^{e}	-
8	1	0.1	-	-	0.20 ± 0.01^{g}	0.24 ± 0.02^{j}	0.35 ± 0.02^{ed}	greenish compact
9	2	0.1	-	-	0.87 ± 0.07^{de}	0.92 ± 0.05^{g}	1.24 ± 0.03^{b}	greenish compact
10	3	0.1	-	-	1.22 ± 0.02^{b}	1.28 ± 0.06^{d}	1.58 ± 0.02^{a}	greenish compact
11	4	0.1	-	-	1.05 ± 0.05^{c}	1.44 ± 0.04^{b}	1.58 ± 0.21^{a}	greenish compact
12	1	0.5	-	-	0.28 ± 0.01^{f}	0.31 ± 0.02^{i}	0.5 ± 0.02^{c}	greenish compact
13	2	0.5	-	-	0.87 ± 0.01^{de}	0.99 ± 0.02^{f}	1.26 ± 0.02^{b}	greenish compact
14	3	0.5	-	-	1.33 ± 0.03^{a}	1.34 ± 0.03^{c}	1.59 ± 0.03^{a}	greenish compact
15	4	0.5	-	-	1.127 ± 0.03^{c}	1.5 ± 0.03^{a}	1.52 ± 0.02^{a}	brownish compact
16	-	-	0.2	-	0^{h}	0^{k}	0^{e}	-
17	-	-	0.4	-	0^{h}	0^{k}	0^{e}	-
18	-	-	0.6	-	0^{h}	0^{k}	0^{e}	-
19	-	-	-	1	0^{h}	0^{k}	0^{e}	-
20	-	-	-	2	0^{h}	0^{k}	0^{e}	-
21	-	-	-	3	0^{h}	0^{k}	0^{e}	-
22	-	-	-	4	0^{h}	0^{k}	0^{e}	-

The distinct letters within the same column indicate significant differences as determined by Duncan's multiple-range test, $p < .05$. BA, benzyladenine; NAA, naphthaleneacetic acid; TDZ, thidiazuron

Table 2 Effects of growth media on shoot regeneration from callus and multiple shoot production of *Stephania pierrei* diels

PGRs (mg/L)						Shoot regenerated from callus		Multiple shoot culture	
No.	BA	TDZ	Kinetin	NAA	Meta-topolin	Average shoot number	Average shoot length (cm)	Average shoot number	Average shoot length (cm)
1	0	-	-	-	-	0^{j}	0^{l}	1.00 ± 0.00^{o}	$2.74 \pm 0.25^{i-l}$
2	1	-	-	-	-	1.40 ± 0.55^{hi}	$3.72 \pm 0.37^{d-g}$	6.80 ± 0.84^{c}	6.54 ± 0.76^{c}
3	2	-	-	-	-	2.80 ± 0.84^{ef}	3.60 ± 1.14^{efg}	$4.80 \pm 0.84^{d-i}$	4.08 ± 0.26^{gh}
4	3	-	-	-	-	7.40 ± 1.14^{a}	$4.20 \pm 0.39^{b-e}$	$5.00 \pm 0.71^{d-h}$	4.10 ± 0.45^{gh}
5	4	-	-	-	-	2.20 ± 0.45^{fg}	$3.00 \pm 0.21^{g-j}$	2.46 ± 0.38^{lmn}	3.14 ± 0.29^{ijk}
6	5	-	-	-	-	1.60 ± 0.55^{hi}	2.36 ± 0.59^{j}	1.00 ± 0.00^{o}	$1.26 \pm 0.49^{o-t}$
7	-	0.2	-	-	-	0^{j}	0^{l}	2.40 ± 0.42^{lmn}	0.36 ± 0.09^{t}
8	-	0.4	-	-	-	0^{j}	0^{l}	4.00 ± 0.71^{hij}	0.35 ± 0.10^{t}
9	-	0.6	-	-	-	0^{j}	0^{l}	3.00 ± 1.00^{jkl}	0.36 ± 0.09^{t}
10	-	0.8	-	-	-	0^{j}	0^{l}	1.40 ± 0.55^{no}	0.38 ± 0.11^{t}
11	1	0.2	-	-	-	1.12 ± 0.18^{i}	2.74 ± 0.43^{hij}	$4.78 \pm 0.41^{d-i}$	$2.44 \pm 0.46^{i-n}$
12	1	0.4	-	-	-	0^{j}	0^{l}	5.76 ± 0.25^{d}	$1.86 \pm 0.23^{l-q}$
13	1	0.6	-	-	-	0^{j}	0^{l}	$5.00 \pm 0.53^{d-i}$	$1.50 \pm 0.10^{n-s}$
14	1	0.8	-	-	-	0^{j}	0^{l}	$4.92 \pm 0.24^{d-i}$	$2.48 \pm 0.36^{j-m}$
15	2	0.2	-	-	-	0^{j}	0^{l}	$4.50 \pm 0.46^{f-i}$	3.20 ± 0.26^{ijk}
16	2	0.4	-	-	-	0^{j}	0^{l}	$5.20 \pm 0.18^{d-h}$	$2.44 \pm 0.27^{j-n}$
17	2	0.6	-	-	-	0^{j}	0^{l}	5.60 ± 0.42^{def}	$2.04 \pm 0.71^{l-p}$
18	2	0.8	-	-	-	0^{j}	0^{l}	$4.92 \pm 0.26^{d-i}$	0.44 ± 0.09^{t}
19	3	0.2	-	-	-	0^{j}	0^{l}	4.30 ± 0.35^{ghi}	$1.00 \pm 0.00^{q-t}$
20	3	0.4	-	-	-	0^{j}	0^{l}	$4.80 \pm 0.58^{d-i}$	$1.00 \pm 0.00^{p-t}$
21	3	0.6	-	-	-	0^{j}	0^{l}	$5.40 \pm 0.37^{d-g}$	$0.50 \pm 0.71^{o-t}$
22	3	0.8	-	-	-	0^{j}	0^{l}	5.72 ± 0.30^{de}	$0.38 \pm 0.00^{q-t}$
23	-	-	1	-	-	0^{j}	0^{l}	1.00 ± 0.00^{o}	$2.80 \pm 0.84^{i-l}$
24	-	-	2	-	-	1.40 ± 0.55^{hi}	2.58 ± 0.58^{ij}	2.50 ± 0.50^{imn}	$2.20 \pm 0.84^{k-o}$
25	-	-	3	-	-	2.40 ± 0.55^{fg}	3.10 ± 0.53^{ghij}	1.00 ± 0.00^{o}	$1.54 \pm 0.46^{m-r}$
26	-	-	1	0.1	-	2.20 ± 0.00^{fg}	0^{l}	1.60 ± 0.55^{mno}	5.02 ± 0.69^{ef}
27	-	-	2	0.1	-	2.80 ± 0.00^{ef}	0^{l}	1.60 ± 0.89^{mno}	5.94 ± 0.43^{cde}
28	-	-	3	0.1	-	3.60 ± 0.84^{c}	0.80 ± 0.19^{k}	1.00 ± 0.00^{o}	3.08 ± 1.01^{ijk}
29	1	-	1	0.1	-	3.20 ± 0.45^{cde}	$4.30 \pm 0.84^{a-e}$	1.00 ± 0.00^{o}	$1.02 \pm 0.11^{q-t}$
30	1	-	2	0.1	-	3.40 ± 0.55^{cd}	5.02 ± 0.99^{a}	2.40 ± 0.55^{lmn}	3.22 ± 0.23^{hij}
31	1	-	3	0.1	-	2.40 ± 0.84^{fg}	4.50 ± 0.50^{abc}	$4.50 \pm 0.85^{f-i}$	$2.62 \pm 0.46^{i-l}$
32	2	-	1	0.1	-	3.00 ± 0.55^{de}	4.66 ± 1.09^{abc}	2.96 ± 0.23^{kl}	5.54 ± 1.11^{def}
33	2	-	2	0.1	-	1.80 ± 0.55^{gh}	3.40 ± 0.55^{fgh}	$4.50 \pm 1.11^{f-i}$	0.33 ± 0.11^{t}
34	2	-	3	0.1	-	4.40 ± 0.71^{b}	$4.46 \pm 1.21^{a-d}$	2.40 ± 0.55^{lmn}	0.52 ± 0.06st
35	3	-	1	0.1	-	4.80 ± 0.84^{b}	4.82 ± 0.75^{abc}	$5.10 \pm 0.74^{d-h}$	$0.88 \pm 0.29^{q-t}$
36	3	-	2	0.1	-	1.00 ± 0.55^{i}	4.74 ± 0.99^{abc}	2.28 ± 0.49^{lmn}	0.80 ± 0.29^{rst}
37	3	-	3	0.1	-	1.00 ± 1.10^{i}	4.88 ± 1.03^{ab}	2.20 ± 0.84^{lmn}	0.38 ± 0.08^{t}
38	-	-	-	-	0.5	1.00 ± 0.00^{i}	3.58 ± 0.51^{efg}	9.60 ± 1.52^{b}	6.10 ± 1.27^{cd}
39	-	-	-	-	1	1.00 ± 0.00^{i}	$4.08 \pm 0.72^{c-f}$	13.40 ± 2.30^{a}	7.80 ± 1.30^{b}
40	-	-	-	-	1.5	1.00 ± 0.00^{i}	$4.20 \pm 0.45^{b-e}$	$5.00 \pm 1.58^{d-h}$	8.80 ± 2.28^{a}
41	-	-	-	-	2	1.00 ± 0.00^{i}	$3.00 \pm 0.71^{g-j}$	3.80 ± 1.30^{ijk}	5.22 ± 0.81^{def}
42	-	-	-	0.2	0.5	1.00 ± 0.00^{i}	2.54 ± 0.51^{ij}	4.40 ± 1.14^{ghi}	4.76 ± 0.83^{fg}
43	-	-	-	0.2	1	1.00 ± 0.00^{i}	3.24 ± 0.89^{ghi}	$4.60 \pm 1.14^{e-i}$	5.26 ± 0.29^{def}
44	-	-	-	0.2	1.5	1.00 ± 0.00^{i}	$3.20 \pm 0.45^{g-j}$	2.60 ± 0.55^{lm}	5.54 ± 0.95^{def}
45	-	-	-	0.2	2	1.00 ± 0.00^{i}	2.60 ± 0.55^{ij}	2.20 ± 0.45^{lmn}	3.50 ± 1.02^{hi}

The distinct letters within the same column indicate significant differences as determined by Duncan's multiple-range test, $p < .05$. PGRs, plant growth regurators; BA, benzyladenine; NAA, naphthaleneacetic acid; TDZ, thidiazuron

following the induction of shoots from calli derived from the tuber and the subsequent multiple shoot inductions from a single shoot. Each shoot was transferred to MS, ½ MS, and ¼ MS media, which were free of PGR, for inducing adventitious roots. The root induction conditions were identical to those used for shoot induction. Each root induction treatment included three replicates, with 10 shoots per replicate. Following a 45-day period of rooting induction, the number and length of roots and rooting rate were measured. The experiment included ten replicates, with one explant per replicate.

Acclimatization

Rooted plants were cultivated in plastic containers filled with autoclaved sand, covered with a clear plastic cap, and watered daily with sterile water for one week. Subsequently they were watered daily with Hoagland solution [34] for 30 days under shade conditions. The rooted plantlets were acclimated in plastic containers filled with a substrate composed of coconut coir, soil, and sand at a 1:1:1 ratio. Finally, well-rooted plants that were efficiently regenerated in vitro underwent acclimatization and were transferred to field environments. Acclimatization of substrates is essential for the effective transition of in vitro-rooted plantlets to ex vitro conditions.

Detection of phytochemicals

Crude extract preparation

Tuber flesh without peel, peel, flesh with peel, and 30-day-old callus derived from the tuber of *S. pierrei* Diels were oven-dried at 40 °C for 24 h. The dry samples were ground in a mechanical grinder to obtain a homogenous powder. Two grams of each sample were placed in 20 mL of ethanol and sonicated for 20 min for extraction. The samples were filtered through a Whatman paper grade 1 filter, and ethanol was removed by evaporation at 40 °C and frozen dry (FreeZone – 50 °C, Labconco). The extracted extracts were stored at – 20 °C, and used for HPLC, gas chromatography–mass spectrometry (GC–MS) analysis, and antioxidant measurement.

Detection and measurement of certain alkaloid contents by using HPLC

Two grams of each freeze-dried crude extract powder were individually added to 25 mL of ethanol and subjected to sonication for 30 min, followed by filtration using a 0.45 µm filter. The injection volume was 10 µL. Cepharanthine (CAS No. 5481-49-2), tetrandrine (CAS No. 518-34-3), and fangchinoline (CAS No. 436-77-1) were purchased from Sigma (Aldrich) and used as the standards. The concentration of each standard was 1 mg/mL. An Agilent 1260 Infinity II Prime HPLC system (Hitachi Chromaster 5420 UV-VIS Detector; Tokyo, Japan) was used. An HPLC chromatogram was obtained at 254 nm. The HITACHI LaChrom C18-AQ (5 µm), 250 × 4.6 mm column was applied together with the mobile phase, which was 0.1% v/v trifluoroacetic acid (TFA) in water (A) and acetonitrile (B) with a 0.8 mL/min flow rate. All experiments were performed in triplicates.

Identification of phytochemicals in callus and tuber tissue by using GC–MS

The products were analyzed using a gas chromatograph (BRUKER 450GC series) equipped with an Rtx-5MS capillary column and a mass spectrometer detector. The sample was filtered through a nylon filter (0.45 µm) before the analysis. The temperature of the column was 60 °C and maintained for 5 min. The temperature was then increased to 200 °C at a rate of 10 °C/min and maintained for an additional 3 min. Finally, the temperature was increased to 280 °C at a rate of 5 °C/min and maintained for 20 min. The injection temperature was 250 °C and that of the ion source of the detector was programmed to 230 °C.

Gene expression analysis by using quantitative real-time polymerase chain reaction

qRT-PCR was used to examine the expression patterns of the coclaurine N-methyltransferase (*CNMT*), norcoclaurine synthase (*NCS*), and 6-O-methyltransferase (*6OMT*) genes in the tuber and callus. Total RNA was isolated from the tuber flesh without peel, peel, tuber flesh with peel, and calli derived from tubers using the method described by Laksana and Chanprame [35]. Reverse transcription of RNA was performed using an Invitrogen 1st Strand cDNA Synthesis Kit. The accuracy of quantification was confirmed by comparing the expression levels of the three genes to that of gene coding actin, a reference transcript [34]. MEGA11 was used to generate specific primers for *6OMT*, *CNMT*, and *NCS* for real-time PCR (Supplementary Table 1).

PCR experiments were conducted using a reaction mixture (20 µL), which included 500 ng of template cDNA, 1× SensiFAST SYBR No-ROX mix buffer (Bioline Reagent Ltd.), and primers (at concentrations of 0.4 µM). A BIO-RAD® CFX96 TouchTM Real-Time PCR apparatus was used to conduct the PCR amplification procedure. PCR was conducted with an initial denaturation at 95 °C for 30 s, followed by 40 cycles of denaturation at 94 °C for 5 s each, annealing for 15 s for each primer (at 52 °C, 59 °C, and 57 °C for the *6OMT*, *CNMT*, and *NCS* genes, respectively), and extension at 72 °C for 10 s. Three biological and technical replicates were analyzed for each sample. The $2^{-\Delta\Delta Cq}$ approach was used to quantify the levels of gene expression [36].

Antioxidant measurements

Determination of total phenol content

A solution containing 10 mg of crude extract obtained from tuber flesh without peel was diluted with 1 mL of ethanol, and a similar procedure was followed to obtain solutions using peel, tuber flesh with peel, and callus. Subsequently, 100 µL of Folin-Ciocalteu reagent (Merck KGaA, Darmstadt, Germany) with a concentration of 50% was added to the 100 µL of the diluted crude extract was combined with 2 mL of sodium carbonate after 2 min. This was followed by addition of 2 mL of sodium carbonate after a duration of 2 min. The mixture was then incubated at room temperature for 30 min in the dark. Subsequently, the absorbance at 750 nm was measured [37] using a micro-plate reader (model Infinite, M200 Pro®, Switzerland). The experimental configuration included a negative control (ethanol) and a positive control (gallic acid). To determine phenolic content, a standard curve was constructed using standard amounts of gallic acid (Merck). The results were presented as milligrams of gallic acid per milligram of dry weight.

Determination of DPPH scavenging percentage

A series of solutions with different concentrations of the extract obtained from tuber flesh without peel, peel, tuber flesh with peel, and callus (25, 50, 150, 250, 500, 750, and 1,000 mg/L) was prepared by diluting 10 mg/mL solutions of the extracts. Subsequently, 100 µL of each solution was combined with 200 µL of DPPH solution. The mixture was incubated at room temperature for 16 min in the dark, and its absorption at 517 nm was measured using a UV-30 spectrophotometer. The experimental configuration included a negative control (ethanol) and a positive control (ascorbic acid). The results were reported as milligram equivalents of ascorbic acid per milligram of dry weight. Subsequently, the data were calculated as IC50 values. The amount of DPPH discoloration resulting from free radical scavenging was determined using the following equation:

$$\text{DPPH Scavenging}\,\% = [(\text{Absorbance of DPPH} - \text{Absorbance of sample})/\text{Absorbance of DPPH}] \times 100$$

Determination of ferric reducing antioxidant power (FRAP)

A series of diluted solutions were made using 200 µL of the extract obtained from tuber flesh without peel, peel, tuber flesh with peel, and callus at different concentrations (25, 50, 150, 250, 500, 750, and 1,000 mg/L) by diluting a 10 mg/mL solution of the extracted material. Subsequently, 3.9 mL of FRAP reagent was added to each solution, which was then mixed and incubated at 37 °C for 30 min. The freshly prepared FRAP reagent consisted of 300 mM acetate buffer at pH 3.6, 10 mM tripyridyltriazine in 40 mM HCl, and 20 mM $FeCl_3$ $6H_2O$ in a volumetric ratio of 10:1:1 [38]. Subsequently, the absorbance of each solution was measured at 595 nm using a micro-plate reader. A standard curve was generated using $FeSO_4$ solutions with concentrations ranging from 0.0025 to 0.02 mg/mL.

ABTS radical cation scavenging activity assay

First, 14 mM ABTS solution in water and a 4.9 mM potassium persulfate ($K_2O_8S_2$) solution were mixed in a 1:1 ratio, resulting in the production of ABTS radical cations ($ABTS^+$). The resulting solution was incubated at 27 °C for 16 h, which was sufficient to achieve consistent absorbance at 734 nm. Following incubation, the solution was diluted with ethanol until it achieved an initial absorbance value of 0.7 ± 0.005 at 734 nm. To analyze the test samples, 950 µL of $ABTS^+$ reagent was combined with 50 µL of the sample or standard of varying concentrations (25, 50, 150, 250, 500, 750, and 1,000 mg/L). Absorbance was measured at 734 nm after 6 min using a micro-plate reader. An experimental setup was established with the negative control, $ABTS.^+$, and the positive control, quercetin. Quantitative data are reported as milligram equivalents of quercetin per milligram of dry weight. A standard curve was constructed by analyzing quercetin solutions at concentrations ranging from 0.62 to 32 mg/L. The $ABTS^+$ scavenging inhibition capability of the extract was determined by applying the following equation and compared with that of quercetin [39]:

$$\%\text{inhibition} = [(\text{Absorbance of quercetin} - \text{Absorbance of sample})]\,/(\text{Absorbance of control})] \times 100$$

Measurement of total flavonoid content

The method described by Lopez et al. [40] was employed to determine the total flavonoid content in the tuber flesh without peel, peel, tuber flesh with peel, and callus of *S. pierrei* Diels. Firstly, 100 µL (1 mg/mL) from each extracted sample was added to 0.3 mL of 5% $NaNO_2$ and 4 mL of distilled water. The solution was allowed left undisturbed for 5 min at room temperature, following which a 0.3 mL aliquot of a solution containing 10% aluminum chloride was added. The mixture was maintained at room temperature for 6 min and then thoroughly mixed with 2 mL of 1 N NaOH. The mixture was diluted with 3.3 mL of distilled water. The absorption at 415 nm was determined using a spectrophotometer. The results of the calibration curve using quercetin were expressed as milligrams of quercetin equivalent per gram of dried weight (mg QE/g DW).

Measurement of total alkaloid content

The methodology established by Zaree et al. [41] was adopted to quantify alkaloid content, with some modifications. Briefly, the extract samples were supplemented

Fig. 2 (See legend on next page.)

(See figure on previous page.)

Fig. 2 Micropropagation of *Stephania pierrei* Diels: (**A**) initiation of callus from tuber on MS salts together with 3 mg/L BA and 0.1 mg/L NAA; (**B**) proliferation of callus on the same medium as (A); (**C**) plant regeneration through callus in MS + 3 mg/L BA; (**D**) plant regeneration through callus in MS + 1 mg/L BA + 2 mg/L kinetin + 0.1 mg/L NAA; (**E**-**G**) multiple shoot production on MS salts and different concentrations of BA or meta-topolin (mT), (**E**) 1.0 mg/L BA, (**F**) 0.5 mg/L mT, (**G**) 1.0 mg/L mT; (**H**) rooted plant on ½ MS; (**I**) 45 days after transferring to pot, (**J**) a small tuber after 2 months in pot; (**K**) 5 months old plant after transferring to pot. Scale bar = 1 cm

with 500 µL of bromocresol green solution (69.8 mg bromocresol green with 3 mL of 2 N NaOH and 5 mL distilled water), 500 µL of phosphate buffer solution (2 M sodium phosphate, pH 2, and 0.2 M citric acid) at pH 4.7, and 1000 µL of chloroform. The resulting mixture was thoroughly mixed. The aforementioned steps were conducted without the inclusion of berberine which was used as the standard. The liquid-phase absorption was measured using a spectrophotometer at a wavelength of 470 nm. To determine the total alkaloid content of the extract, a standard curve was constructed using the concentration (mg) of berberine per mL of the extract.

All antioxidant measurement experiments were carried out in triplicates.

Statistical analysis

Statistical analyses were performed using the R statistical package R [42]. Significant variations among the means ($p < .05$) were identified using Duncan's multiple-range test (DMRT).

Results

Callus induction

All organs except leaves were found to have the ability to produce calluses. BA in concert with NAA promoted callus induction, whereas TDZ and 2,4-D alone did not. The tubers cultivated in a culture medium containing MS basal salts, supplemented with 3 mg/L of BA and 0.5 mg/L of NAA, obtained a maximum callus weight of 1.59 ± 0.031 g. Calli displayed greenish and compact structures. The highest initiation of callus at a weight of 1.5 ± 0.032 g was observed when the node was cultured in MS medium supplemented with 4 mg/L BA and 0.5 mg/L NAA. Calli in this case exhibited brown coloration. For the shoot tip, the MS medium supplemented with 3 mg/L BA and 0.5 mg/L NAA resulted in the highest callus initiation, weighing 1.33 ± 0.032 g. However, MS medium supplemented with the same quantity of BA and NAA did not initiate callus growth in leaves. Among the three explant types, the tubers had the highest callus weights (Table 1). Nonetheless, the weight of the calli derived from the tubers cultured in this medium exhibited no significant difference when compared to the calli generated in MS medium supplemented with 3–4 mg/L BA, whether used alone or in combination with 0.1 or 0.5 mg/L NAA (Fig. 2A, B).

Plant regeneration through callus, multiple shoot production, root induction, and acclimatization

The 30-day-old calluses derived from tubers cultured on MS salts supplemented with 3 mg/L BA and 0.5 mg/L NAA were subcultured in shoot regeneration medium containing various types and concentrations of PGRs. The plant began to develop from the callus approximately 30 d after being transferred to the medium. Specifically, MS supplemented with 3 mg/L BA exhibited the greatest number of shoots (7.40 ± 1.14) (Fig. 2C, Supplementary Fig. 1). The shoot lengths obtained from media containing 1–3 mg/L BA, 1–3 mg/L kinetin, and 0.1 mg/L NAA were statistically similar but superior to other combinations of PGRs. However, MS with 1.0 mg/L BA, 2 mg/L kinetin, and 0.1 mg/L NAA displayed the highest shoot length of 5.02 ± 0.99 cm (Fig. 2D). Neither BA combined with TDZ nor TDZ alone could induce differentiation of callus into shoots, except for the formula containing BA at 1 mg/L combined with TDZ at 0.2 mg/L, which was able to induce shoot formation from callus (Table 2). Two weeks after transferring a single shoot onto each medium to produce multiple shoots, callus formation was observed at the cut ends of the shoots. About 1–2 weeks later, small shoots began to appear on these calluses. The formula adding mT produced the highest average shoot quantity and shoot length. The MS containing 1 mg/L mT produced the highest average shoot numbers of 13.4 ± 2.30 (Fig. 2G), while the MS containing 1.5 mg/L mT produced the height on average shoot length (8.8 ± 2.28 cm) (Table 2, Supplementary Fig. 2). When roots were induced using MS, ½ MS, and ¼ MS, the highest number and length of roots was observed in the case of ½ MS produced at 3.33 ± 0.3 roots and 3.40 ± 0.13 cm, respectively (Fig. 3). This was significantly different from the number and length of roots obtained using other media. When randomly selected healthy plantlets derived from ½ MS were transferred to a substrate consisting of soil, sand, and coconut coir, all plantlets survived and subsequently produced healthy tubers (Fig. 2H-K).

Detection and measurement of certain alkaloid contents using HPLC

Fangchinoline and cepharantine contents were highest in the peel at 503.52 ± 25.64 and 504.35 ± 8.97 µg/g DW, respectively, which were statistically different from other types of tissues. Tetrandrine was most abundant in tubers with peel at 1506.09 ± 22.30 µg/g DW (Table 3).

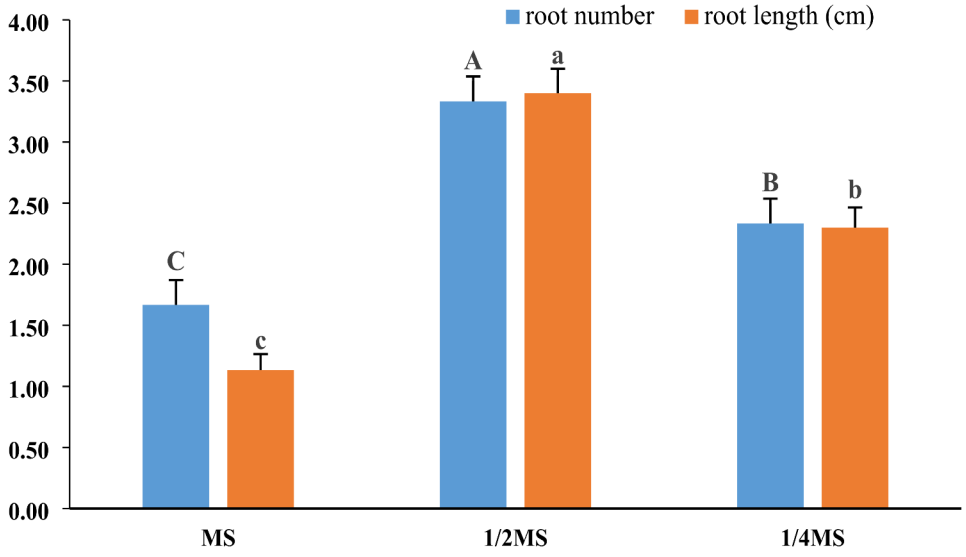

Fig. 3 Root number and root length of regenerated shoots of *Stephania pierrei* Diels induced in three different strengths of MS-salt medium. The different letters on the boxes with the same color indicate that they are significantly different according to DMRT, $p < .05$

Table 3 The contents of the essential alkaloids, namely fangchinoline, cepharantine, and tetrandrine, in the callus, tuber flesh without Peel, tuber flesh with Peel, and Peel of *Stephania pierrei* diels

Type of tissue	Compound (µg/g DW)		
	Fangchinoline	Cepharantine	Tetrandrine
Callus	307.45 ± 13.75^{d}	373.15 ± 11.84^{b}	1324.98 ± 21.05^{d}
Tuber without peel	430.37 ± 28.51^{c}	303.12 ± 4.58^{c}	1402.13 ± 60.98^{c}
Peel	503.53 ± 25.65^{a}	504.35 ± 8.97^{a}	1506.09 ± 22.30^{a}
Tuber with peel	496.34 ± 86.03^{b}	377.80 ± 24.45^{b}	1445.95 ± 41.69^{b}

The distinct letters within the same column indicate significant differences as determined by Duncan's multiple-range test, $p < .05$. DW; dried weight

Notably, calli also contained all three essential alkaloids (fangchinoline, cepharantine, and tetrandrine) although the amount of alkaloids did not reach a maximum. We did not detect any of the three alkaloids in the leaf tissues.

Identification of phytochemicals in callus and tuber tissue by using gas chromatography–mass spectrometry (GC–MS)

The GC–MS profile of the extract obtained from the callus and tuber flesh with peel of *S. pierrei* Diels, revealed the presence of various phytochemical components. The phytochemicals found only in calli included furfural, butyrolactone, 2(5 H)-furanone, 2-furancarboxaldehyde, 5-methyl-; 2,4-dihydroxy-2,5-dimethyl-3(2 H)-furan-3-one; 2 H-pyran-2,6(3 H)-dione; 4-oxopentanoic acid; 3-furancarboxylic acid, methyl ester; pyrimidine, 4-chloro-5-ethoxy-2-methyl-; 5-hydroxymaltol; 5-hydroxymethylfurfural; cirsiumaldehyde, and linolenic acid. However, certain phytochemicals were found only in the tubers. These included 2-methoxy-4-vinylphenol, 4-hydroxybenzyl alcohol; 2,5-dimethoxy-4-methyl-benzaldehyde; 2-hydrazino-7-phenoxytropone, and beta-sitosterol. Some substances were present in both the calli and tubers. These included 4 H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-4(H)-pyran-4-one; n-hexadecanoic acid; linoleic acid; octadecanoic acid; campesterol; and stigmasterol (Table 4, Supplementary Fig. 3).

qRT-PCR for gene expression analysis

The relative expression levels of *CNMT*, *NCS*, and, *6OMT* genes, which have been associated with the synthesis of isoquinoline alkaloids, were assessed in the callus, tuber flesh without peel, peel, and tuber flesh with peel of *S. pierrei*. The highest degree of expression of the *CNMT* gene was observed in the peel, followed by callus and tuber with peel. Conversely, the relative expression level of the gene was the lowest in the tuber flesh without peel. The highest level of relative expression of the *NCS* gene was in the peel, followed by the callus, tuber flesh with peel, and tube flesh without peel. The peel had the highest degree of expression of the *6OMT* gene, followed by the tuber flesh with peel, callus, and tuber flesh without peel (Fig. 4).

Determination of antioxidant activity

The antioxidant potential of the callus, tuber flesh without peel, peel, and tuber with peel was determined by DPPH scavenging percentage, FRAP, and ABTS radical cation scavenging activity assays. DPPH and ABTS assays were performed to determine the half-maximal inhibitory concentration (IC50), and the results were compared with those of ascorbic acid and quercetin, respectively. The peel sample exhibited the lowest IC50 values for DPPH and ABTS, measuring 500.58 ± 0.102

Table 4 Characteristics of selected phytochemical components obtained from the ethanol extract of callus and tuber flesh with Peel using GC–MS analysis

No.	Compound	%Area		Retention time	
		Callus	Tuber[1]	Callus	Tuber[1]
1	Furfural	4.777	-	3.681	-
2	Butyrolactone	0.542	-	6.207	-
3	2(5 H)-Furanone	0.405	-	6.28	-
4	2-Furancarboxaldehyde, 5-methyl-	7.124	-	7.054	-
5	2,4-Dihydroxy-2,5-dimethyl-3(2 H)-furan-3-one	1.091	-	7.592	-
6	4 H-Pyran-2,6(3 H)-dione	0.450	-	8.141	-
7	4-oxopentanoic acid,	0.216	-	9.17	-
8	3-Furancarboxylic acid, methyl ester	2.068	-	9.835	-
9	4 H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-4(H)-pyran-4-one	14.596	16.985	11.307	11.073
10	Pyrimidine, 4-chloro-5-ethoxy-2-methyl-	1.985	-	11.452	-
11	5-Hydroxymaltol	1.408	-	11.994	-
12	5-Hydroxymethylfurfural	34.011	-	13.049	-
13	2-Methoxy-4-vinylphenol	-	5.639	-	13.546
14	4-hydroxybenzyl alcohol	-	22.388	-	15.526
15	2,5-Dimethoxy-4-methylbenzaldehyde	-	6.7120	-	16.843
16	2-Hydrazino-7-phenoxytropone	-	12.838	-	19.493
17	n-Hexadecanoic acid	1.170	4.093	21.856	21.821
18	Cirsiumaldehyde	22.457	-	22.946	-
19	Linoleic acid	4.582	5.935	25.18	25.126
20	Linolenic acid	2.070	-	25.309	-
21	Octadecanoic acid	0.414	2.168	25.544	25.508
22	Campesterol	0.284	9.051	42.604	42.636
23	Stigmasterol	0.351	9.440	43.296	43.328
24	beta-Sitosterol	-	4.752	-	44.674

[1] Tuber implies tuber flesh with peel

and 406.26 ± 8.83 µg/mL, respectively. However, there were no significant differences in the average IC50 values for DPPH and ABTS in any tissue type. The results of the FRAP assay indicate that the peel exhibited its maximum effectiveness at 196.5 ± 1.74 µg $FeSO_4$/mg sample, and it was differently significant from the IC50 of other tissue types. The lowest FRAP value was exhibited by tuber flesh without peel(Table 5).

Determination of total flavonoid, total phenol, and total alkaloid content

The peel sample had the highest total flavonoid concentration, which was significantly different from the callus, tuber flesh without peel, and tuber flesh with peel samples. The callus showed the highest total alkaloid content; however, there were no significant differences among the tissue types. The peel sample had the highest total phenolics content, which was significantly different from that of the rest of the tissue types (Table 6).

Discussion

S. pierrei Diels, widely distributed in Thailand and Indo-China, has historically been used for its medicinal properties [43] and as an ingredient in cuisines. It is a popular indoor ornamental plant. Numerous plants have been consequently felled in the forests in order to satisfy the increasing demand for this species. In this study, we investigated the application of in vitro propagation of *S. pierrei* Diels and explored the potential pharmaceutical properties of this indigenous plant species. We found that the concentration and type of PGRs in the culture medium and the plant parts play crucial roles in determining the initiation of explant development in vitro. The highest callus weight derived for all explants, except the leaves, resulted from media containing 3–4 mg/L BA together with 0.1 or 0.5 mg/L NAA. The synergistic effects of BA and NAA are crucial for callus formation and organ development in vitro [44]. These PGRs are used for callus induction and proliferation, resulting in the differentiation of calli into tubers [45]. The inclusion of BA and NAA in culture media enhances tuber formation in plants such as *Pogostemon cablin* and *Amorphophallus* species [46]. The combined effect of these PGRs not only boosts the development of callus but also accelerates the pace at which tubers develop, eventually leading to successful tuber formation. Hence, the deliberate use of BA and NAA in tissue culture methods may efficiently induce tuberization in diverse plant species.

Studies have been conducted on callus induction from node, tuber, and leaf explants of different plant species. Studies on *Talinum paniculatum* and *Pueraria tuberosa* have shown effective callus formation and shoot growth in leaf explants. Of these two species, *T. paniculatum* exhibits a higher rate of shoot formation in leaf explants [47, 48]. In contrast, yam species, such as *Dioscorea tokoro*, can create calluses and regenerate plantlets from tuber discs [49]. Furthermore, studies on potato plants have shown that nodal explants are more successful than leaf segments in inducing callus formation and promoting the growth of new shoots [50]. Callus initiation can also be achieved using tuber cuttings, internodes, and leaf disc explants, as reported in *Plectrantus esculentus*. Recent studies have explored callus induction using shoot tip explants across various plant species, focusing on optimizing culture media and plant growth regulator (PGR) combinations to enhance regeneration. For instance, shoot tip culture of *Cochlospermum religiosum* on MS medium supplemented with 1.0 mg/L BAP and 0.5 mg/L IAA effectively induced light green, fragile calli with the capability of plant regeneration [51]. Similarly, shoot tips of *Gymnema sylvestre* cultured on MS medium

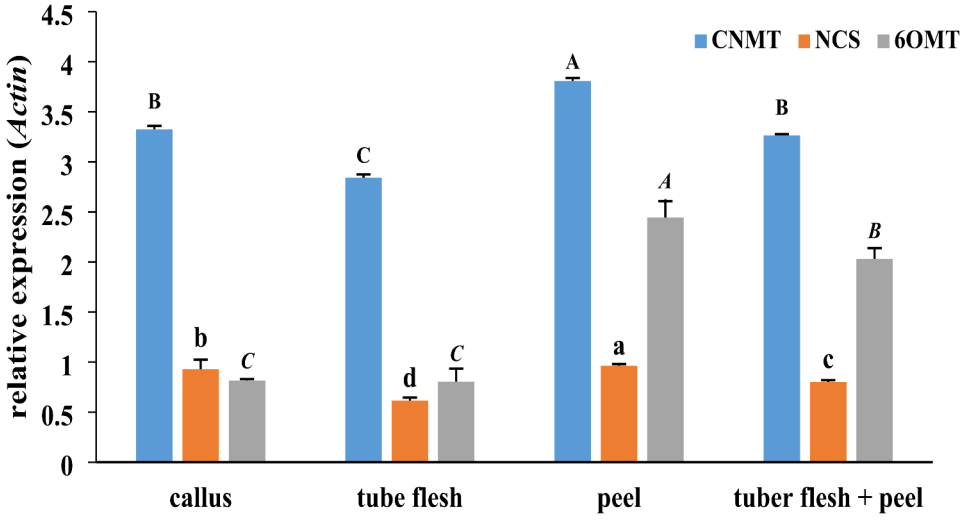

Fig. 4 Relative gene expression levels of coclaurine N-methyltransferase (*CNMT*), norcoclaurine synthase (*NCS*), and 6-O-methyltransferase (*6OMT*) in the callus, tuber flesh without peel, peel, and tuber flesh with peel of *S. pierrei* Diels. The values presented are the means of three replicates ± SE. Different letters on the boxes with the same color denote statistically significant differences among distinct types of tissue, according to Duncan's multiple-range test, $p < .05$

Table 5 Antioxidant activities of callus, tuber flesh without peel, peel, and tuber flesh with peel extracts from *S. pierrei* as determined using the ABTS, DPPH, and FRAP assays

Sample	DPPH assay (IC50 µg/mL)	ABTS assay (IC50 µg/mL)	FRAP assay (µg $FeSO_4$/mg sample)
Callus	664.6023 ± 0.894	421.69 ± 0.78	153.08 ± 1.56^{C}
Tuber flesh without peel	698.311 ± 0.140	421.46 ± 0.88	135.71 ± 1.11^{D}
Peel	500.58 ± 0.102	406.26 ± 8.83	196.5 ± 1.74^{A}
Tuber flesh with peel	557.0283 ± 0.939	412.67 ± 6.69	164.60 ± 1.071^{B}
F-test	ns	ns	*

ns: non-significant difference; *: significant difference at $p < .05$ according to Duncan's multiple-range test. FRAP, ferric reducing antioxidant power

Table 6 Total flavonoid, total phenolic, and total alkaloid contents in the callus, tuber flesh without peel, peel, and tuber flesh with Peel extracted from *S. pierrei*

Sample	Total flavonoid content (g QE/100 g extract)	Total phenolic content (mg GAE/100 g extract)	Total alkaloid content (mg berberine/g extract)
Callus	2.032 ± 0.06^{B}	109.39 ± 0.10^{C}	76.98 ± 0.57
Tuber flesh without peel	2.041 ± 0.05^{B}	131.80 ± 0.10^{B}	72.59 ± 0.78
Peel	3.104 ± 0.01^{A}	195.33 ± 0.47^{A}	73.17 ± 0.15
Tuber flesh with peel	2.063 ± 0.01^{B}	132.26 ± 0.64^{B}	72.21 ± 0.25
F-test	*	*	ns

ns: non-significant difference; *: significant difference at $p < .05$ according to Duncan's multiple-range test

supplemented with 2.0 mg/L 2,4-D exhibited profuse callus formation within 2–3 weeks. Subsequent transfer to media containing BAP or TDZ facilitated shoot morphogenesis [52]. However, callus induction varies with the plant part used for the process, and each explant type requires distinct ideal conditions for callus formation [53]. In the present study, shoot tips, nodes, and tubers demonstrated effective callus induction, among which tubers proved to be the most effective explants for callus production.

Further, we found that calli cultured on MS medium with 3 mg/L BA exhibited the highest average shoot number. Successful evidence of shoot regeneration from calluses using BA has been provided for several plant species. Experimental investigations on *Aconitum bucovinense*, *Dendrocalamus brandisii*, and *Haworthia* sp. have consistently demonstrated the efficacy of BA in stimulating shoot growth in callus cultures [45, 54–56].

Achieving multiple shoot cultures was successful using mT independently at a rate of 1 mg/L, which resulted in a maximum average number of shoots. mT is an aromatic cytokinin that shows considerable promise for plant tissue culture as it plays a role in stimulating the growth of new shoots, prolonging the aging process, and improving the reproduction of shoots [57, 58]. Suzuki et al. [19] induced multiple shoots from calluses obtained from young stems of *S. cepharantha* with 11 plants per callus. In the present study, the highest shoot length was recorded at an mT concentration of 1.5 mg/L. mT shows significant potential for producing shoots of *Crinum brachynema* (Amaryllidaceae) in vitro, with the best shoot regeneration rate observed for medium supplemented with 5.0 µM mT, achieving a frequency of 11.1

shoots [59]. Jayaprakash et al. [60] developed an in vitro regeneration technique in which *Oxystelma esculentum* nodal explants were cultured on MS medium supplemented with BA and mT and reported that mT is more efficient than BA in shoot induction and development from the existing meristems of explants. mT has been effectively used in the in vitro propagation of various plant species as well as in improving the growth of shoots in regenerated plants. Compared to other cytokinins such as BA, mT offers benefits such as faster shoot growth, easier adaptation to new conditions, and enhanced root development in plants [57, 61].

In the present study, root induction was the highest, both in terms of quantity and length, when ½ MS medium was used. This is consistent with the findings of Asmono et al. [62], who observed that modifying the MS medium led to the highest root number, root length, and plantlet length in stevia (*Stevia rebaudiana* Bertoni), with the ½ MS medium being the most effective in promoting an increase in root length. Alteration of MS media is more effective in enhancing plantlet length compared to full media and does not show a significant difference compared to ½ MS. Root induction is hormone-independent. Root induction is a straightforward procedure because auxin, the naturally occurring plant hormone, plays an essential role in controlling many elements of root growth and structure [63, 64]. In the current study, the MS PGR-free medium successfully induced root growth. This suggests that the regenerated shoots have sufficient endogenous auxin to initiate root growth. Additionally, the choice of substrate significantly influences the morphological features and physiological parameters of plantlets during the acclimatization process [65]. We found that the substrate comprising coconut coir, soil, and sand at a 1:1:1 ratio was efficient in successful acclimatization, which emphasizes the importance of selecting appropriate acclimatization substrates for the effective establishment of in vitro-derived plantlets in greenhouse and field environments.

In this study, we also used GC–MS analysis to identify the phytochemical components in callus tissues and compared them with those observed in the tuber flesh of *S. pierrei* Diels. Among medicinal plants, *S. pierrei* Diels is a primary source of several bioactive chemicals [7, 8, 12]. Their use is likely to have originated from forested areas. However, the present quantity is insufficient to meet the demand, which is causing significant damage to the long-term viability and health of the species. Phytochemical production via callus culture is a sustainable method that can avoid the need to harvest plants from the wild. Of the 24 phytochemicals studied in the present study, 19 and 11 were found in calli and tubers with peels, respectively. The 13 phytochemicals found only in calli, including 2(5 H)-furanone derivatives, particularly chiral sulfones with terpene moieties, show promise for inhibiting biofilm formation by bacteria. These phytochemicals exhibit significant efficacy against *Bacillus subtilis* and *Staphylococcus aureus* [66]. Additionally, 4-oxopentanoic acid, another important substance found in calli and also known as 4-oxo-2-pentenoic acid, demonstrates versatile therapeutic potential; it exhibits an antagonistic effect on ileal smooth muscle excitation caused by acetylcholine, inhibits normal isolated intestinal function, and suppresses intestinal propulsion in spleen-deficient mice and castor oil-induced diarrhea [67]. 4 H-Pyran-2,6(3 H)-dione is a widely recognized component of various natural and synthetic products and exhibits a diverse array of biological activities, including its ability to protect the liver, inhibit tumor growth, reduce inflammation, act as an antioxidant, relax muscles, increase urine production, mimic estrogen, prevent blood clotting, prevent antifungal and viral infections, lower body temperature, combat tuberculosis and HIV, and relieve seizures and pain [68]. 5-Hydroxymaltol has antioxidant and anti-inflammatory properties [69]. 2-Methoxy-4-vinylphenol (2M4VP) is a naturally occurring compound in red wine that possesses anti-inflammatory properties [70]. 4-hydroxybenzyl alcohol demonstrates potential therapeutic benefits for neuroprotection and epilepsy prevention, making it a compound of interest for further research and development [71]. The six phytochemicals were found in both calli and tubers, including octadecanoic acid, campesterol, and stigmasterol. Octadecanoic acid derivatives have antimicrobial properties against bacteria and fungi [72]. Campesterol, a phytosterol with diverse biological activities, has potential as an antiarthritic and anti-inflammatory agent, inhibiting arthritis development [73]. Stigmasterol is a phytosterol that has several pharmacological properties, such as anti-diabetic, anti-inflammatory, or anti-tumor properties [74]. Of the five phytochemicals found only in the tubers, one was 4-hydroxybenzyl alcohol, which has neuroprotective properties and demonstrates antiepileptogenic actions by slowing down the course of seizures [71].

In the present study, HPLC analysis revealed important alkaloids, including tetrandrine, fangchinoline, and cepharanthine, in tuber flesh without peel, peel, tuber with peel, and callus of *S. pierrei* Diels. Tetrandrine, fangchinoline, and cepharantine are produced during the biosynthesis of isoquinoline alkaloid biosynthesis [75]. Several alkaloids are found in the plants in the genus *Stephania*, such as *S. tetrandra* (tetrandrine, fangchinoline, and cepharanthine) [76, 77]; *S. rotunda* (cepharanthine and fangchinoline) [78]; *S. japonica* (cepharanthine) [79]; and *S. cephalantha* (cepharanthine) [80]. In the present study, the peel contained the highest concentrations of fangchinoline and cepharanthine, whereas the tuber with peel contained the highest concentration of tetrandrine.

We found that the concentration of cepharantine in callus was at a high level, comparable to that found in tuber with peel, whereas the concentrations of fangchinoline and tetrandrine in callus were lower than those in tuber with peel. Meanwhile, the expression of the genes related to the biosynthesis of these three compounds in callus, compared to tuber with peel, varied: *CNMT* was at a similar level, *NCS* was higher, and *6OMT* was lower. This suggests that if the expression of all three genes in callus could be upregulated, there would be a possibility of enhancing the biosynthesis and accumulation of these compounds, particularly cepharantine, in callus. This implies that calli possess a substantial capability to synthesize these vital alkaloids that can mitigate long-term contamination by bacteria, fungi, and heavy metals. Furthermore, the production of these alkaloids via callus culture can be performed year-round under controlled environmental conditions. The therapeutic potential of these vital alkaloids for the treatment of diseases can be further enhanced by increasing their quantities in calli.

The principal secondary metabolites of the *Stephania* genus are isoquinoline alkaloids. Semwal et al. [7] comprehensively documented the chemical composition and pharmaceutical properties of members of the *Stephania* genus. Tetrandrine effectively suppresses the growth of hepatocellular carcinoma and enhances the responsiveness to sorafenib by targeting the PI3K/AKT/mTOR signaling pathway, triggering apoptosis, arresting the cell cycle in the G1 phase, and inhibiting cell migration [81]. Fangchinoline effectively suppresses the growth of breast cancer cells [82, 83]. A growing body of evidence suggests that tetrandrine alkaloids exhibit promising anticancer characteristics, with a particular focus on their potential for treating lung cancer [84]. The antineoplastic effects of cephalanthine have been observed in numerous cancer cell lines. In contrast, Liu et al. [85] reported that cepharanthine is effective against nasopharyngeal cancer (NPC) and has been widely employed in the management of numerous acute and chronic conditions. However, previous studies have demonstrated its antiviral effectiveness against the influenza A virus (IAV) and coronaviruses, such as severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) [85, 86].

Benzylisoquinoline alkaloids (BIA), including tetrandrine, fangchinoline, and cepharantine, are the principal bioactive compounds utilized in the isoquinoline-alkaloid pathway to produce alkaloids. Biosynthesis of BIA is regulated by several enzymes, including 6OMT, CNMT, and NCS [87, 88]. We found that the genes for these three enzymes exhibited the highest levels of expression in the peel, which were significantly different from other tissue types. We also found that tetrandrine, fangchinoline, and cepharantine were the most prevalent in the peel. Further, the callus exhibited higher levels of gene expression than the tuber flesh, which highlights the ability of calli as a substitute source for the production of these alkaloids instead of natural tubers. Sawant and Dhabe [89] examined the bioactive chemicals in *Ceropegia bulbosa* var. *bulbosa* using HR-LCMS and revealed that the presence of these compounds is comparable in both the tubers and calli. However, they noted that some chemicals, such as Validamycin A and Flurazepam, were found only in the callus. Jayalakshmi et al. [90] compared callus induction from *Coccinia indica* leaves with that from tuber extracts and reported that callus is an important source of phytochemicals, antibacterial agents, antioxidants, and α-amylase inhibitors.

There have been numerous studies showing that plants in the genus *Stephania* contain compounds with antioxidant activity in various parts of the plant, such as the leaves of *S. japonica* [91], the aerial parts (except flowers) and tuber of *S. rotunda* [78], *S. wightii* [92], *S. abyssinia* [93] and the tuber of *Stephania glabra* [94]. In the present study, the peel of the tuber exhibited the highest flavonoid and phenolic content, followed by tuber flesh with peel, tuber flesh without peel, and callus. However, no significant difference was observed in the total alkaloid content, although the callus exhibited the highest total alkaloid concentration. DPPH and ABTS radical scavenging assays showed that the peel had the highest antioxidant efficiency owing to the lowest IC50 values, but this was not statistically different from the antioxidants of callus, tuber flesh without peel, and tuber with peel. Furthermore, the peel had the highest FRAP value, which was significantly different from the FRAP values of the other plant parts and calluses. Even though the peel has the most effective antioxidant properties, there is a limited quantity of peel. However, peeling the tubers was not essential. Based on the experiment's findings, it was shown that the callus is as effective as the tuber component. Hence, the use of calluses is an alternative that reaps advantages in terms of both treatment and propagation.

Conclusions

To the best of our knowledge, this is the first study to investigate in vitro propagation of *S. pierrei* Diels. We found that tuber flesh was the most efficient explant for callus induction during the micropropagation of the species. These tubers should be cultured on MS salts containing 3–4 mg/L BA and 0.1 or 0.5 mg/L NAA. Shoot regeneration was accomplished using the MS medium containing 3 mg/L BA. To produce multiple shoots, it was necessary to subculture a single shoot onto an MS medium containing 1 mg/L mT. Subsequently, each shoot should be transferred to a half-strength MS salt medium to induce root growth. This is also the first study to report that the callus of this species not only contains the

essential alkaloids (fangchinoline, cepharantine, and tetrandrine) but also contains some pharmaceutical properties and phytochemicals that are not found in the tubers. Successful in vitro propagation of this native species can contribute to the effective management of this species in the wild, preventing its extinction. Our significant findings indicate that the callus of this species contains many kinds of essential phytochemicals possessing pharmaceutical properties, suggesting that the callus can also be used for the mass production of such phytochemicals on an industrial scale. However, to enhance such alkaloids production from callus culture, further researches on elicitor application, manipulation of culture environments, modification of culture medium, and production through bioreactor systems are needed to be established.

Abbreviations

2,4-D	2,4-Dichloropheoxy acetic acid
6OMT	6-O-methyltransferase
ABTS	2,2′-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid)
BA	N6-Benzyladenine
CNMT	Coclaurine N-methyltransferase
DPPH	2,2-Diphenyl-1-picrylhydrazyl
DW	Dry weight
FRAP	Ferric reducing antioxidant power
GA	Gibberellic acid
GC-MS	Gas chromatographymass spectrometry
HPLC	High Performance Liquid Chromatography
IBA	Indole-3-butyric Acid
MS	Murashige and Skoog medium
mT	Meta-topolin
NAA	Naphthalene acetic acid
NCS	Norcoclaurine synthase
PGRs	Plant grow regulators
PPFD	Photosynthetic photon flux density
qRT-PCR	Quantitative real-time polymerase chain reaction
TDZ	Thidiazuron

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

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Author contributions

CL planned and designed of the research, performed the experiments, data analyzed, wrote the manuscript. OS performed the experiments and data analyzed. SN performed the experiments, and data analyzed. SC planned and designed of the research, wrote the manuscript.

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Data availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

Permission to collect *Stephania pierrei* Diels specimens from the Faculty of Agricultural Technology area at Burapha University's Sakaeo Campus was granted by the Acting Dean of the Faculty of Agricultural Technology, Burapha University, Sakaeo Campus, Thailand, on January 8, 2021.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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