In Vitro Cell. Dev. Biol.—Plant 38:451–455, September–October 2002
DOI: 10.1079/IVP2002322

1054-5476/02 $10.00+0.00

A RAPID PROTOCOL FOR *IN VITRO* MICROPROPAGATION OF *LEPIDIUM SATIVUM* LINN. AND ENHANCEMENT IN THE YIELD OF LEPIDINE

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(Received 17 October 2001; accepted 30 April 2002; editor W.-Y. Soh)

Summary

Lepidium sativum L., commonly known as 'garden cress', possesses variable proportions of benzylcyanide and benzylisothiocynate which contribute towards its activity against *Bacillus subtilis* and *Micrococcus pyogenes*. The plant is also used as an antifertility and antiovulatory drug. Various juvenile (cotyledonary leaves, hypocotyl, radicle) as well as mature explants (leaf, shoot apex, nodal segments) callused on Murashige and Skoog's medium (MS) supplemented with α-naphthaleneacetic acid (NAA) + N^6-benzyladenine (BA) + casein hydrolyzate (CH). Regeneration from hypocotyl callus and nodal segments occurred after NAA/BA was replaced with indole-3-acetic acid (IAA)/kinetin (Kn). Lepidine was monitored at regular intervals. Significant amounts of lepidine was detected in *in vitro*-regenerated plants obtained from juvenile and mature explants. The yield, however, was variable, depending upon the source and type of explant used. High lepidine was detected in 8-wk-old hypocotyl callus. Amongst regenerants, maximum lepidine was obtained from the plantlets at the vegetative stage.

Key words: garden cress; hypocotyl; nodal segment; regeneration; IAA–kinetin interaction; lepidine content.

Introduction

Industrialization coupled with urbanization is constantly putting pressure on natural resources. Due to the depletion of habitat and ruthless collection pressure, medicinal plants are disappearing fast from the wild. Hence, conservation of germplasm becomes an urgent requirement. Plant tissue culture technique holds great promise for micropropagation, conservation, and enhancement of the natural levels of valuable secondary plant products. *Lepidium sativum* Linn. (Brassicaceae) is an important source of bioactive compounds. The plant, considered to have originated in the highland regions of Ethiopia and Eritrea, is of great medicinal value in the treatment of asthma, coughs, and bleeding piles (Anonymous, 1962). Leaves, being mildly stimulant and diuretic, are used in the scorbutic diseases and hepatic disorders (Anonymous, 1962). The medicinal properties of roots have been ascribed for secondary syphilis and tenesmus. Seeds are used widely as a rubefacient, galactogogue, tonic, laxative, aphrodisiac, and diuretic (Anonymous, 1962). Abortifacient property (Anonymous, 1962) and antiovulatory functions of the seeds have also been described (Jamwal and Anand, 1962; Malhi and Trivedi, 1972; Kamboj and Dhawan, 1982; Satyavati, 1984). *In vitro* propagation of medicinal plants holds tremendous potential for the production of high-quality plant-based medicine (Murch et al., 2000).

Despite its potential therapeutic values, the plant has not received the attention it deserves. The main objective of this study was to evaluate the levels of lepidine at different stages of growth and differentiation in both *in vivo* and *in vitro* plants. In addition, the effect of auxin/cytokinin interactions on the morphogenic response of juvenile (hypocotyl) as well as mature (nodal segment) explants was monitored to formulate a reliable regeneration protocol. As the plants are annual, *in vitro* propagules could prove fruitful as a continuous source of raw material for valuable medicinal compounds like benzylisothiocynate, benzylcyanide, and an alkaloid (lepidine), which possesses antifertility activity (Pande et al., 1999). Conventionally, seeds, which may not yield uniform levels of lepidine, are being used as a source material.

Materials and Methods

Medium and Establishment of Cultures. Five hundred mature seeds of *Lepidium sativum* Linn. procured from the herbal garden at Jamia Hamdard were washed with 0.2% Cetrimide (*N*-cetyl-*N*,*N*,*N*-trimethyl ammonium bromide) (ICI, India) for 5 min and rinsed three times with distilled water. Seeds were then disinfected with 10% NaOCl for 10 min and 0.1% $HgCl_2$ for 5 min followed by 70% alcohol for 1 min, and rinsed thoroughly with sterile distilled water. One hundred seeds were also presoaked in autoclaved gibberellic acid [GA_3; 2.0 mg l^{-1} (5.78 μ*M*)] solution for 12 h. All the seeds were allowed to germinate aseptically on MS (Murashige and Skoog, 1962) basal medium gelled with 0.6% agar. Mature nodal segments obtained from field-grown plants were additionally treated with 0.5% streptomycin sulfate (Sigma) + 1% Bavistin (BASF, India) for 30 min, and implanted on MS medium supplemented with two concentrations of indole-3-acetic acid [IAA; 0.2 mg l^{-1} (1.14 μ*M*) and 0.5 mg l^{-1} (2.85 μ*M*)] and kinetin [Kn; 2.0 mg l^{-1} (9.28 μ*M*) and 5.0 mg l^{-1} (23.2 μ*M*)]. Juvenile explants (cotyledonary leaves, hypocotyl, and radicle segments) excised from 17-d-old seedlings (~5 cm long) and mature explants (leaf, shoot apex, and nodal segment) from field-grown plants were inoculated horizontally on MS supplemented with

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TABLE 1

LEPIDIUM SATIVUM: EFFECT OF NAA–BA INTERACTIONS ON CALLOGENIC RESPONSE IN VARIOUS EXPLANTS

NAA, mg l^{-1} (μM)	BA, mg l^{-1} (μM)	Callogenic response (%)					
		In vitro explants			*In vivo* explants		
		Cotyledonary leaves (mean ± SE)	Hypocotyl segments (mean ± SE)	Radicle segments (mean ± SE)	Leaf segments (mean ± SE)	Shoot apex (mean ± SE)	Nodal segments (mean ± SE)
0.5 (2.78)	1.0 (4.68)	No response	11.0 ± 0.2	No response	No response	No response	No response
0.5 (2.78)	2.0 (8.86)	No response	16.0 ± 0.1	No response	No response	No response	No response
0.5 (2.78)	5.0 (22.15)	No response	23.7 ± 0.3	No response	No response	No response	No response
1.0 (5.37)	1.0 (4.43)	5.4 ± 0.3	37.7 ± 0.3	No response	No response	No response	No response
1.0 (5.37)	2.0 (8.86)	19.0 ± 0.8	59.0 ± 0.3	No response	10 ± 1.8	11 ± 2.0	No response
1.0 (5.37)	5.0 (22.15)	24.0 ± 0.3	75.2 ± 0.2	8.9 ± 0.1	10 ± 2.0	12 ± 1.3	No response
2.0 (10.74)	1.0 (4.43)	38.0 ± 0.3	86.0 ± 0.3	17.0 ± 0.3	32 ± 1.8	No response	47 ± 2.0
2.0 (10.74)	2.0 (8.86)	44.9 ± 0.3	91.0 ± 0.2	27.0 ± 1.7	71 ± 1.3	64 ± 1.7	80 ± 1.9
2.0 (10.74)	5.0 (22.15)	61.3 ± 0.2[a]	100 ± 0.0[a]	42.0 ± 0.1	90 ± 1.2	82 ± 1.8	No response
5.0 (26.85)	1.0 (4.43)	14.9 ± 0.5	50.4 ± 0.5	12.0 ± 0.3	No response	No response	No response
5.0 (26.85)	2.0 (8.86)	No response	15.7 ± 0.3	No response	No response	No response	No response
5.0 (26.85)	5.0 (22.15)	No response	No response	No response	No response	No response	No response

Data are the mean ± SE of two independent experiments of each treatment with 24 cultures.
[a] Green compact callus.

different levels of α-naphthaleneacetic acid (NAA) and N^6-benzyladenine (BA) (see Table 1).

For each set of experiments, percent cultures showing shoot regeneration, average number of shoots per culture and their height were scored at weekly intervals over a period of 3 wk. All the cultures were maintained at 25 ± 2°C under 16 h photoperiod provided by fluorescent tubes and incandescent bulbs with a total irradiance of 57 $\mu mol\, m^{-2}\, s^{-1}$ (Philips, India) at the culture level.

Regenerated shoots (~ 10-wk-old) were rooted on growth regulator-free MS medium fortified with glutamine [100 mg l^{-1} (684 μM)] that prevented leaf abscission also. Plantlets (7–8 cm tall) were transferred fortnightly to basal medium (with a gradual reduction in MS salt concentration to half, one-fourth and one-eighth of normal, respectively). Fully-grown plantlets (12 cm) were finally transferred to plastic pots (7.0 × 10.0 cm) with potting mixture. Pots containing a mixture of soilrite and quartz (3:1) proved best. The pots were initially maintained for 2 wk in a growth chamber at 25–30°C under 12 h light (conditions same as above) cycle for acclimatization before transfer to the field.

Quantification of Lepidine. High performance liquid chromatography (HPLC). Reverse phase HPLC (Perkin Elmer) was performed with methanol:water (45:55) as the mobile phase at a flow rate of 1 $cm^3\, min^{-1}$ maintained by a binary pump (Perkin Elmer-LC 250). Separation was done in C_{18} peco sphere column with a functional surface of octadecyl dimethyl silane (partical size: 3 μm, column dimensions: 83 × 4.6 mm). A diode array UV detector (Perkin Elmer-LC 235) was used at 278–279 nm, sensitivity 0.5 AUFS (Absorbance Unit Full Scale) and a band width of 5. The plotting and peak area calculations were accomplished with an integrator (Perkin Elmer-LCI 100).

Standard curve of lepidine. A standard sample of lepidine was obtained from Sigma Chemicals (St. Louis, MO). Stock solution (1 mg ml^{-1}) of lepidine was prepared in methanol. From the stock solution, dilutions of 2–20 μl were taken in duplicates and analyzed independently by HPLC and a standard curve was plotted between concentration and peak area. The injected quantities (2–20 μl) and peak areas showed good linearity.

Sample preparation for estimation of lepidine. For each sample, 100 g (see Table 4) was powdered with a mortar and pestle, and defatted with petroleum ether (40–60°C). The defatted samples were then separately dried and extracted with methanol. The methanolic extracts were evaporated under reduced pressure to dryness and dissolved in 10 cm^3 methanol. A fixed amount (25 μl) of each sample was subjected to HPLC. Reproducibility of the method was achieved by injecting samples in triplicate into the HPLC and the mean values were recorded. The areas of the relevant peaks of each sample were interpolated with the standard curve to determine the quantity of lepidine (in mg g^{-1}). The experiment was repeated twice.

Results and Discussion

In this study we report a protocol for continuous high-frequency shoot multiplication and plantlet production in *Lepidium sativum*. All the explants (~ 5 mm), when cultured on MS supplemented with NAA, BA, and casein hydrolyzate (CH) (Table 1), initiated callus at the cut end within 18 d. Further proliferation occurred all over the surface on MS + NAA [1.0 mg l^{-1} (5.37 μM)] + BA [5.0 mg l^{-1} (22.15 μM)] + CH (1000 mg l^{-1}).

A wide variation in callus production was observed, when various combinations of NAA and BA (Table 1) were used in the MS medium; MS + NAA [2.0 mg l^{-1} (10.74 μM)] + BA [5.0 mg l^{-1} (22.15 μM)] + CH (1000 mg l^{-1}) proved best for hypocotyl explants and produced compact, green regenerating callus with several tiny shoot bud-like structures in 100 ± 0.0% of cultures (Fig. 1*A*).

Since the callus derived from leaves, shoot apices and nodal segments (mature explants), and cotyledonary leaves and radicles (juvenile explants) showed low morphogenic potentiality (3–10%, respectively) (data not shown), further regeneration studies were focused only on hypocotyl callus. The hypocotyl callus (~ 100 mg) upon transfer to MS containing IAA [0.25 mg l^{-1} (1.42 μM) and 0.5 mg l^{-1} (2.85 μM)] + Kn [2 mg l^{-1} (9.28 μM) and 5 mg l^{-1} (23.2 μM)] + adenine [Ad; 20 mg l^{-1} (148 μM)] + asparagine [Asp; 100 mg l^{-1} (666 μM)] + Glu [100 mg l^{-1} (684 μM)] + CH (1000 mg l^{-1}), regenerated shoots after 2 wk (Fig. 1*B*). The highest regeneration frequency (100 ± 0.0%) with the highest number of shoots per culture (25.8 ± 0.2) of 2.5 ± 0.2 cm height, however, occurred on MS containing 0.5 mg l^{-1} (2.85 μM) IAA + 3.0 mg l^{-1} (13.92 μM) Kn (Fig. 1*C*). Even a slight deviation in this combination led to a poor response (Table 2). This indicates a

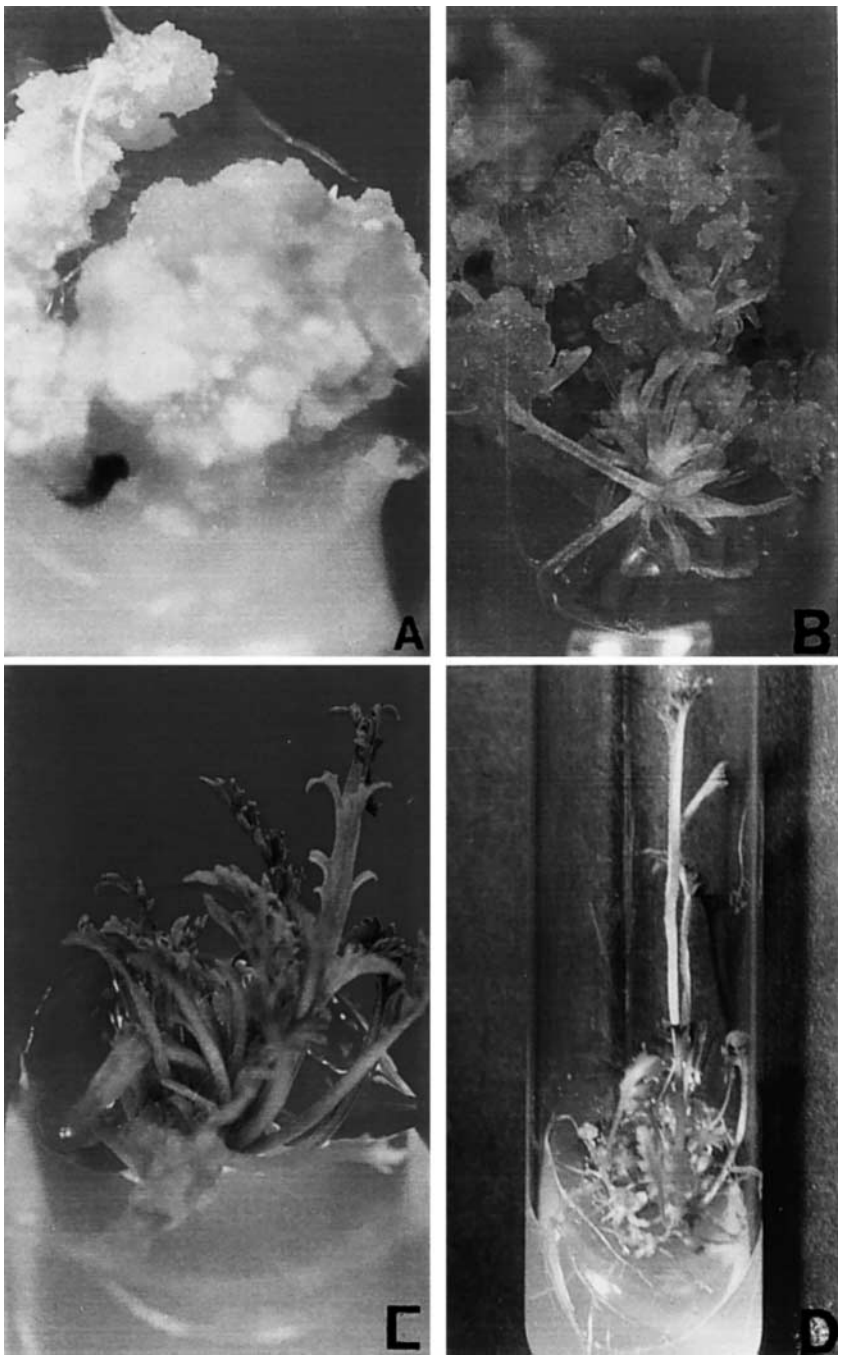

FIG. 1. *Lepidium sativum*: regeneration through hypocotyl callus. *A*, Compact and green callus on MS + NAA [2.0 mg l^{-1} (10.74 μM)] + BA [5.0 mg l^{-1} (22.15 μM)] + CH (1000 mg l^{-1}); 4 wk old. *B*, Emergence of shoots on MS + IAA [0.5 mg l^{-1} (2.85 μM)] + Kn [3.0 mg l^{-1} (13.92 μM)] + Ad [20 mg l^{-1} (148 μM)] + Asp [100 mg l^{-1} (666 μM)] + Glu [100 mg l^{-1} (684 μM)] + CH (1000 mg l^{-1}); 6 wk old. *C*, Growth of shoots; 14 wk old. *D*, Fully developed plantlet on MS + Glu [100 mg l^{-1} (684 μM)]; 18 wk old.

very precise requirement of IAA and Kn for maximum morphogenic response in *Lepidium sativum*. Similarly, in *Ammi majus* (Purohit et al., 1995; Pande et al., 2002) and *Brassica* spp. (Evans et al., 1981) reasonable regeneration in callus cultures occurred on MS supplemented with 0.5 mg l^{-1} (2.32 μM) and 5.0 mg l^{-1} (23.2 μM) Kn and low concentrations of IAA or NAA.

Mature nodal segments cultured on MS with IAA [0.2 mg l^{-1} (1.14 μM)] and Kn [2.0 mg l^{-1} (9.28 μM) and 5.0 mg l^{-1} (23.2 μM)] differentiated four or five shoots per explant after 15 d. The shoots attained a height of 9 ± 0.20 cm within 6 wk (see Table 3). When 0.2 mg l^{-1} (1.14 μM) IAA was used with 2.0 mg l^{-1} (9.28 μM) Kn, the regeneration frequency was 58.3 ± 0.88% and reached 75 ± 1.73% with 5.0 mg l^{-1} (23.2 μM) Kn. The shoots were taller (10 ± 0.41 cm) and showed profuse branching at the

TABLE 2

LEPIDIUM SATIVUM: EFFECT OF IAA–Kn INTERACTIONS ON MORPHOGENIC RESPONSE OF HYPOCOTYL CALLUS GROWN ON MS + IAA + Kn + Ad [20 mg l^{-1} (148 μM)] + Asp [100 mg l^{-1} (666 μM)] + Glu [100 mg l^{-1} (684 μM)] + CH (1000 mg l^{-1})

IAA, mg l^{-1} (μM)	Kn, mg l^{-1} (μM)	% Cultures showing regeneration (mean ± SE)	Shoots per culture (mean ± SE)	Height of regenerated shoots (cm) (mean ± SE)
0.25 (1.42)	2.0 (9.28)	80.0 ± 1.0	5.0 ± 0.1	0.7 ± 0.1
0.25 (1.42)	3.0 (13.92)	84.5 ± 0.4	8.0 ± 0.1	1.2 ± 0.1
0.25 (1.42)	4.0 (18.56)	76.0 ± 1.0	9.5 ± 0.2	1.8 ± 0.1
0.25 (1.42)	5.0 (23.20)	72.5 ± 2.9	7.6 ± 0.2	1.5 ± 0.1
0.25 (1.42)	10.0 (46.40)	77.0 ± 4.0	2.3 ± 0.1	0.9 ± 0.1
0.50 (2.85)	2.0 (46.40)	95.0 ± 2.8	11.1 ± 0.3	1.7 ± 0.2
0.50 (2.85)	3.0 (46.40)	100 ± 0.0	25.8 ± 0.2	2.5 ± 0.2
0.50 (2.85)	4.0 (46.40)	90.0 ± 1.2	20.6 ± 0.5	1.8 ± 0.1
0.50 (2.85)	5.0 (46.40)	85.0 ± 1.5	15.5 ± 0.3	1.5 ± 0.1
0.50 (2.85)	10.0 (46.40)	10.0 ± 2.6	8.6 ± 1.7	0.7 ± 0.1

Data are the mean ± SE of two independent experiments of each treatment with 24 cultures.

TABLE 3

LEPIDIUM SATIVUM: EFFECT OF IAA–Kn INTERACTIONS ON THE REGENERATION RESPONSE OF NODAL SEGMENTS ON MS + IAA + Kn + CH (500 mg l^{-1})

IAA, mg l^{-1} (μM)	Kn, mg l^{-1} (μM)	% Regeneration (mean ± SE)	Shoots per culture (mean ± SE)	Height of shoots (cm) (mean ± SE)
0.2 (1.14)	2.0 (9.28)	58.3 ± 0.88	6.5 ± 1.13	9.0 ± 0.88
0.2 (1.14)	5.0 (23.2)	75 ± 1.73	13.6 ± 0.54	10.0 ± 0.41
0.5 (2.85)	2.0 (9.28)	62.3 ± 1.70	8.7 ± 0.23	8.0 ± 0.30
0.5 (2.85)	5.0 (23.2)	42.6 ± 1.45	5.8 ± 0.51	4.9 ± 0.48
1.0 (5.70)	2.0 (9.28)	27.2 ± 1.2	4.0 ± 0.21	2.2 ± 0.10
1.0 (5.70)	5.0 (23.2)	14.3 ± 0.74	2.1 ± 0.04	1.9 ± 0.15

Data are the mean ± SE of two independent experiments of each treatment with 24 cultures.

nodes. Further increase in IAA [0.5 mg l^{-1} (2.85 μM)] with Kn [2.0 mg l^{-1} (9.28 μM) and 5.0 mg l^{-1} (23.2 μM)] reduced the regeneration percentage to 62.3 ± 1.70 and 42.6 ± 1.45, respectively (Table 3).

The promotive effect of cytokinins on callus and root initiation but no impact on shoot formation in *Brassica* spp. (Murata and Orton, 1987) does not corroborate the findings of Skoog (1971) who pointed out that the shoot induction property of Kn is accelerated if used with Ad and low concentrations of IAA or NAA. The present work on *Lepidium sativum* therefore supports the contention of Skoog.

Rooting and transplantation of plantlets to the field is the most important but difficult task in micropropagation (Murashige, 1974). Shoots (~3–4 cm), when transferred to MS + Glu [100 mg l^{-1} (684 μM)] produced prominent and thick roots in 100 ± 0.0% cultures (Fig. 1*D*). Implicitly, the regeneration of plantlets in *Lepidium sativum* strictly follows a two-step process of shoot differentiation followed by rooting. The overall success of tissue culture-raised plants depends upon successful transplantation to the field. The heterotropic mode of nutrition and poorly developed mechanism to control water loss renders micropropagated plants vulnerable to transplantation shock that results in low survival in the field (Hussey, 1986). The acclimatization procedure followed by us involved a gradual depletion of carbon source along with other organic adjuvants coupled with sequentially lowering of the strength of the MS salts. The process ensured acclimatization, including hardening of plantlets; and 65 ± 0.75% of the plants survived through this procedure.

Lepidine content. One of the advantages of tissue culture of medicinal plants, besides the study on growth and differentiation under various chemical milieus, is the availability of higher biomass (callus) that provides an opportunity to assay the yield of secondary metabolites at specific stages of growth and differentiation. It also facilitates the use of promoters and elicitors. Plant cell culture technology can be exploited profitably to ensure the availability of raw material throughout the year and, if the yield can be increased successfully in cultures, bioreactors can be put to use for mass production of the product.

Lepidine estimated at various stages of *in vivo* and *in vitro* growth exhibited variation (Table 4). Synthesis of secondary metabolites has been linked to a certain degree of differentiation of the individual cells. In the present study, among the *in vivo* material, maximum lepidine (7.7 ± 0.13 mg g^{-1} dry weight) was detected in 2-mo.-old (preflowering) field-grown plants. As the plant matured, lepidine content declined gradually, reaching 5.0 ± 0.21 mg g^{-1} dw at the flowering stage (Table 4). Similarly, among the *in vitro* cultures, maximum lepidine (10.4 ± 0.11 mg g^{-1} dw) was obtained in the plantlets at the vegetative stage, and that declined in plantlets at the flower stage (Table 4). In various other medicinal plants also a certain degree of differentiation has been considered

TABLE 4

LEPIDIUM SATIVUM: LEPIDINE CONTENT IN SAMPLES

Samples	Lepidine content ($mg\,g^{-1}$ dw) (mean ± SE)
In vivo	
Seeds	3.7 ± 0.06
Seedlings	4.7 ± 0.07
Leaves	4.2 ± 0.10
Mature plants (vegetative stage)	7.7 ± 0.13
Mature plants (flowering stage)	5.0 ± 0.21
In vitro	
Cotyledonary leaf callus	8.7 ± 0.07
Hypocotyl callus	9.0 ± 0.06
Shoot apex callus	5.3 ± 0.11
Nodal segment callus	4.3 ± 0.16
Regenerants (shoots)	7.4 ± 0.07
Plantlets (vegetative stage)	10.4 ± 0.05
Plantlets (flowering stage)	6.9 ± 0.11

Data are the mean ± SE from two independent experiments each with three replicates.

essential for secondary metabolite biosynthesis (see Srivastava et al., 1993; Datta and Srivastava, 1997; Pande et al., 2000). The yield of secondary metabolites also fluctuates according to various environmental, physiologic, and genetic factors (see Srivastava and Pande, 1998). The present study on *Lepidium* corroborates this fact.

Until now there have been no reports on the regeneration and secondary metabolite assessment at various stages of *in vitro* growth in *Lepidium sativum*. The only earlier report by us (see Saba et al., 2000) deals with the effect of heavy metal stress (Cu and Zn) on regeneration and lepidine content. The present investigation therefore is the first report describing an efficient protocol for *de novo* shoot regeneration from hypocotyl callus and nodal segments of *Lepidium sativum* and the systematic monitoring of lepidine at various stages of growth. Our investigations show that the cultures of *Lepidium sativum* can serve as a potential source of enhanced levels of secondary metabolites under suitable conditions.

Acknowledgments

D.P. is thankful to the Council of Scientific & Industrial Research, New Delhi (CSIR) for awarding a Research Associateship. Mr. Satish Chand helped in typing and formatting the manuscript.

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