BIOLOGIA PLANTARUM 43 (2): 253-256, 2000

# Effect of $ZnSO_4$ and $CuSO_4$ on regeneration and lepidine content in *Lepidium sativum* L.

SABA, D. PANDE, M. IQBAL*, P.S. SRIVASTAVA**

*Centre for Biotechnology and Department of Botany*, Faculty of Science, Hamdard University, New Delhi 110 062, India*

## Abstract

Significant amounts of lepidine was detected in mature and juvenile explants from both *in vivo* and *in vitro* grown plants. The yield, however, was variable depending upon the source and type of explant used. Mature *in vivo* plants at vegetative stage exhibited highest yield. Among all the explants, maximum lepidine was detected after 8 weeks in shoot apex callus on MS medium supplemented with 2 mg $dm^{-3}$ naphthaleneacetic acid and 5 mg $dm^{-3}$ benzylaminopurine. Addition of 900 μM $Zn^{2+}$ or 100 μM $Cu^{2+}$ further enhanced the yield of lepidine.

*Additional key words*: differentiation, *in vitro* growth, medicinal plant, secondary metabolites.

## Introduction

Tissue culture techniques could be used for the micropropagation of medicinally important plants and in enhancing the natural levels of valuable secondary products (Reinert and Bajaj 1977). For example, structure, function and biosynthesis of artemisinin have been studied and worked out through these methods (Bharel *et al.* 1996, Gulati *et al.* 1996a,b). Enhancement in the yield of secondary metabolites has been detected at specific stages of growth and differentiation (Srivastava *et al.* 1993, Purohit *et al.* 1995a,b). Cell growth and secondary metabolites can be manipulated by changing the media and culture conditions.

In spite of great medicinal values of *Lepidium sativum*, there are few reports so far of systematic investigation on its phytochemistry (Khel and Kunjundzić 1985, Srivastava and Pande 1998, Saba *et al.* 1998, 1999), pharmacology and biotechnology (Brunner and Rudiger 1994). Lepidine, a 4-methylquinoline (cincholepidine - $C_{10}H_9N$), molecular mass 143.18 kDa, with bp 261 - 263 has anti-scorbutic properties.

Metal ions such as $Cu^{2+}$, $Mn^{2+}$, $Zn^{2+}$ and $Fe^{2+}$ are essential trace nutrients taking part in redox reactions, structural configuration of several enzymes and nucleic acid metabolism (Zenk 1996). At higher concentration, however, they become strongly poisonous causing inhibition of growth and metabolism and even death of the organism (Zenk 1996). Although, these heavy metals are widely used as microelements, their effective roles on morphogenesis in plants more so in medicinal herbs need extensive elaboration. Since, stress has been implicated in secondary metabolite production, the present work was undertaken to assess the role of metal stress on growth and differentiation as well as on secondary metabolite production in a medicinal plant. There is no equivocal report on the role of heavy metals on the production of secondary metabolites in medicinal herbs. The work clearly demonstrates that the *in vitro* raised plants of *Lepidium* can sustain a certain degree of metal stress and respond by enhanced biosynthesis of lepidine content in the cultures.

## Material and methods

**Preparation of culture medium**: MS (Murashige and Skoog 1962) medium with 3 % sucrose, gelled with 6 g $dm^{-3}$ of agar (*Merck*, New Delhi, India) was used throughout. MS medium was supplemented with auxins,

*Received* 10 May 1999, *accepted* 15 November 1999.
*Abbreviations*: BAP - benzylaminopurine; IAA - indole-3-acetic acid; Kn - kinetin; NAA - 1-naphthaleneacetic acid.
**Author for correspondence; fax: (+91) 11.6988874, e-mail: root@hamduni.ren.nic.in

indole-3-acetic acid (IAA), 1-naphthaleneacetic acid (NAA), and cytokinins, zeatin (Z), benzylaminopurine (BAP) and kinetin (Kn) at various concentrations and combinations. In case of test tubes (150 × 25 mm), 15 $cm^3$ of the medium was dispensed, while in 100 $cm^3$ Erlenmeyer flasks, 40 - 45 $cm^3$ of the medium was dispensed. The culture vessels were plugged with non-absorbent cotton wrapped in single layered muslin cloth. The pH of the medium was adjusted to 5.7 with 0.1 M NaOH or 0.1 M HCl prior to autoclaving.

**Establishment of cultures:** Seeds of *Lepidium sativum* L. procured from the Herbal Garden at Jamia Hamdard were grown in experimental beds. Explants (leaf, shoot apex, nodal segments) obtained from these field-grown plants were surface sterilized by washing with 2 % *Cetrimide* (alkyltrimethylammonium bromide) for 15 min followed by treatment with 0.5 % streptomycin sulphate + 1 % *Bavistin* (carbendazim) for 30 min. After thorough washing with sterile distilled water, they were sequentially treated with 0.1 % $HgCl_2$ for 5 min and 70 % ethyl alcohol for 1 min. The explants were then thoroughly rinsed with sterile distilled water and implanted aseptically on the culture medium.

Effect of metal stress ($ZnSO_4$ or $CuSO_4$) was studied on growth as well as on secondary metabolite production of both organized and unorganized tissues by incorporating 25, 50, 75, 100 μM of $CuSO_4$ or 100, 200, 300, 400, 500, 600, 700, 800, 900 μM of $ZnSO_4$ in MS medium containing IAA, NAA and Kn. Fresh mass (f.m.) and dry mass (d.m.) was calculated after every 4 weeks till 12 weeks. In addition, shoot regeneration, average number of shoots per culture, average shoot length and rooting were also analyzed.

Standard sample of lepidine was obtained from *Sigma Chemicals* (St Louis, USA). The injected quantities and peak areas showed good linearity. 100 g of each sample was powdered in a mortar with pestle and defatted with petroleum ether (40 - 60 °C). The defatted samples were separately dried and extracted with methanol. The methanolic extracts were evaporated under reduced pressure to dryness and dissolved in 10 $cm^3$ methanol. Fixed amount of each sample was subjected to HPLC with a flow rate of 1 $cm^3$ $min^{-1}$ and alkaloid peaks were detected by a UV detector at 278 - 279 nm. The solvent applied was MeOH (pump A) and $H_2O$ (pump B) in the ratio of 45:55. Reproducibility of the method was achieved by injecting duplicate quantities to the HPLC and the mean values were obtained. Lepidine was detected by a *Merck LiChroCART* 125 × 4 mm HPLC $C_{18}$ column, packed with Lichrosphere 100 beads of 5 μm diameter and a functional surface of octadecylsilane (reverse phase) at 278 nm and eluted with MeOH:$H_2O$ (45:55) at a flow rate of 1 $cm^3$ $min^{-1}$. Quantification was done by concentration/peak area ratio (response factor).

## Results

Maximum lepidine [8.7 mg $g^{-1}$(d.m.)] was detected in two-month-old (preflowering) field grown plants. As the plants matured, lepidine content declined gradually reaching 5 mg $g^{-1}$(d.m.) at the flowering stage. The seeds contained 3.7 mg $g^{-1}$(d.m.) of lepidine, which was higher than young leaves 2 mg $g^{-1}$(d.m.).

Leaves, shoot apex and nodal segments of *in vivo* plants were grown on MS medium supplemented with various combinations of NAA and BAP and variable amount of callus was observed. Callus cultures of both, leaves and nodal segments showed best yield on MS + 2 mg $dm^{-3}$ NAA + 5 or 7 mg $dm^{-3}$ BAP. In all subsequent studies, therefore, this medium was used.

In leaf callus on MS + 2 mg $dm^{-3}$ NAA + 5 mg $dm^{-3}$ BAP, after 8 weeks of culture, 9 mg $g^{-1}$(d.m.) lepidine was detected which was maximum among all the stages of cultures analyzed. On higher auxin concentration, lepidine content decreased. The lepidine content in shoot apex callus was slightly less than that of leaf callus. Maximum lepidine content of 8.7 mg $g^{-1}$(d.m.), was observed also after 8 weeks. Nodal segment callus maintained on MS + 2 mg $dm^{-3}$ NAA + 5 mg $dm^{-3}$ BAP showed half lepidine content as compared to leaf callus of the same age.

When 4-week-old calli from all the above explants were transferred to various combinations of NAA/Kn, multiple shoot formation was observed only on MS + 0.1 mg $dm^{-3}$ NAA + 5 mg $dm^{-3}$ Kn. With the initiation of shoot buds, lepidine content declined initially to 2.6 mg $g^{-1}$(d.m.), but after 8 weeks, with further growth of shoots, a gradual rise to 6.8 mg $g^{-1}$(d.m.)] was noticed. After 10 weeks, a sudden drop in lepidine content to 2 mg $g^{-1}$(d.m.)] was observed. Emergence of roots after 12 weeks caused drop in lepidine content to negligible amount [0.9 mg $g^{-1}$(d.m.)]. With the onset of flower buds, the content of lepidine rose to 3 mg $g^{-1}$(d.m.).

When 100 or 200 μM of $ZnSO_4$ was added to the MS medium with 0.1 mg $dm^{-3}$ NAA + 5 mg $dm^{-3}$ Kn, a rapid rise in growth was observed till 8 weeks. A maximum of 4 shoots with normal leaves per culture was recorded and the shoots showed normal growth. At 300 μM $ZnSO_4$ the increase in growth declined. The regenerants developed short internodes with thin leaves at 500 μM $ZnSO_4$. At 900 μM of $ZnSO_4$ toxic effects were visible after 12 weeks. The shoots showed reduced growth with short internodes and thin leaves (Table 1).

Except 25 μM all other concentrations of $CuSO_4$ showed toxic effect after 8 weeks. The regenerants on

Table 1. Effect of addition of $ZnSO_4$ in different concentrations [μM] on fresh mass [g], dry mass [mg], and lepidine content [mg $g^{-1}$(d.m.)] of *Lepidium sativum* regenerants grown on MS medium + 0.1 mg $dm^{-3}$ NAA + 5 mg $dm^{-3}$ Kn for 4, 8 and 12 weeks. Means ± SE, n = 48.

| $ZnSO_4$ | | 4 weeks | 8 weeks | 12 weeks |
|---|---|---|---|---|
| 0 | fresh mass | 0.92±0.40 | 1.02±0.67 | 1.07±0.54 |
| | dry mass | 0.10±0.20 | 0.11±0.29 | 0.13±0.01 |
| | lepidine | 3.90±0.02 | 8.80±0.02 | 1.90±0.02 |
| 100 | fresh mass | 0.75±0.13 | 0.88±0.00 | 0.91±0.06 |
| | dry mass | 0.09±0.01 | 0.08±0.00 | 0.09±0.00 |
| | lepidine | 6.50±0.02 | 8.80±0.03 | 8.30±0.03 |
| 300 | fresh mass | 0.66±0.10 | 0.88±0.01 | 0.91±0.04 |
| | dry mass | 0.07±0.00 | 0.08±0.00 | 0.09±0.00 |
| | lepidine | 2.80±0.02 | 8.80±0.01 | 2.00±0.02 |
| 500 | fresh mass | 0.61±0.07 | 0.72±0.03 | 0.76±0.03 |
| | dry mass | 0.07±0.00 | 0.08±0.00 | 0.09±0.00 |
| | lepidine | 3.80±0.02 | 9.00±0.02 | 7.50±0.03 |
| 700 | fresh mass | 0.58±0.05 | 0.61±0.01 | 0.64±0.00 |
| | dry mass | 0.05±0.00 | 0.05±0.00 | 0.06±0.00 |
| | lepidine | 2.20±0.02 | 8.80±0.02 | 0.30±0.02 |
| 900 | fresh mass | 0.50±0.00 | 0.53±0.02 | 0.57±0.03 |
| | dry mass | 0.04±0.01 | 0.04±0.00 | 0.05±0.00 |
| | lepidine | 8.20±0.02 | 9.90±0.03 | 1.80±0.02 |

25 μM of $CuSO_4$ grew normal even after 16 weeks. A gradual rise in the mass of regenerants was observed till 12 weeks and 4 shoots per culture were recorded after 16 weeks. At 50 μM of $CuSO_4$ the biomass decreased rapidly and the leaves turned yellow and abscised after 12 weeks. $CuSO_4$ at 75 μM showed high toxicity and after 16 weeks caused browning of cultures. 100 μM of $CuSO_4$ proved to be highly toxic (Table 2).

Table 2. Effect of addition of $CuSO_4$ in different concentrations [μM] on fresh mass [g], dry mass [mg], and lepidine content [mg $g^{-1}$(d.m.)] of *Lepidium sativum* regenerants grown on MS medium + 0.1 mg $dm^{-3}$ NAA + 5 mg $dm^{-3}$ Kn for 4, 8 and 12 weeks. Means ± SE, n = 48.

| $CuSO_4$ | | 4 weeks | 8 weeks | 12 weeks |
|---|---|---|---|---|
| 0 | fresh mass | 0.47±0.17 | 0.55±0.17 | 1.83±0.19 |
| | dry mass | 0.41±0.00 | 0.04±0.03 | 0.18±0.05 |
| | lepidine | 2.00±0.03 | 8.80±0.03 | 6.10±0.02 |
| 25 | fresh mass | 0.45±0.14 | 0.53±0.17 | 0.72±0.18 |
| | dry mass | 0.04±0.01 | 0.04±0.14 | 0.08±0.06 |
| | lepidine | 2.70±0.03 | 7.00±0.02 | 7.50±0.02 |
| 50 | fresh mass | 0.46±0.15 | 0.56±0.17 | 0.70±0.10 |
| | dry mass | 0.04±0.01 | 0.04±0.00 | 0.04±0.03 |
| | lepidine | 2.00±0.02 | 6.80±0.02 | 4.10±0.02 |
| 75 | fresh mass | 0.62±0.14 | 0.70±0.16 | 0.66±0.12 |
| | dry mass | 0.05±0.00 | 0.05±0.00 | 0.05±0.01 |
| | lepidine | 3.80±0.02 | 10.00±0.02 | 3.80±0.02 |
| 100 | fresh mass | 0.66±0.21 | 0.47±0.15 | 0.00±0.00 |
| | dry mass | 0.04±0.00 | 0.03±0.00 | 0.00±0.00 |
| | lepidine | 10.20±0.02 | 10.00±0.02 | 4.00±0.02 |

Lepidine content was also monitored in cultures grown on media additionally supplemented with $ZnSO_4$ or $CuSO_4$. After 8 weeks in cultures grown on 75 or 100 μM of $CuSO_4$ lepidine content was 1.2 mg $g^{-1}$(d.m.) higher than in control (Table 2). $ZnSO_4$ also induced enhancement in the yield of lepidine (Table 1).

## Discussion

Micropropagation of medicinal plants can become purposeful only when the yield of useful drug component is maintained or enhanced. The initial optimism for production of secondary metabolites (Ramawat 1995) through plant tissue culture has been somewhat tampered by the observations that cultured plant cells routinely yield very low concentrations of the commercially most important secondary products. The present investigation, however, is suggestive of sufficient yield of lepidine from *Lepidium sativum* cultures. Shimomura *et al.* (1991) have demonstrated the commercially viable high yield of shikonin produced by hairy root cultures of *Lithospermum erythrorhizon*. The product is now being produced on a commercial scale. Also, Purohit *et al.* (1995a,b) reported higher yield of xanthotoxin in *Ammi majus* cultures.

The yield of secondary metabolite fluctuates according to various environmental, physiological and genetical factors (see Smorodin *et al.* 1979, Srivastava and Pande 1998). The present study on lepidine corroborates this fact. A high degree of variation in metabolites in different parts and also stages of plants have been reported by Manot (1987). In *Lepidium sativum*, maximum yield was obtained in mature plants at pre-flowering stage and declined subsequently. Secondary metabolite accumulation has been detected in organised and unorganised cultures of several other medicinal plants as well (Srivastava *et al.* 1993). Young plantlets of *Lepidium sativum* yielded less lepidine than mature plantlets (Saba *et al.* unpublished). The result supports the earlier findings of Constabel *et al.* (1974) and Cordüan (1975).

There are studies that have evaluated the yield of secondary metabolites in the parent plant and in the *in vitro* grown cultures. Rarely, low yielding plants have yielded high quantities of secondary metabolites in tissue cultures (Constabel 1990). The present investigations elucidate that the cultures of *Lepidium sativum* can serve

as a potential source of secondary metabolites under suitable conditions. Cu proved to be more effective than Zn in enhancing the yield of lepidine. Apparently, the *in vitro* cultures of *Lepidium sativum* could serve as a continuous supply of lepidine.

## References


Bharel, S., Gulati, A., Abdin, M.Z., Srivastava, P.S., Jain, S.K.: Structure, biosynthesis and function of artimisinin. - Fitoterapia **67**: 387-402, 1996.

Brunner, H., Rudiger, W.: On the expression of several *Lhc* genes in garden cress *(Lepidium sativum)*. – Z. Naturforsch. **49c**: 802-810, 1994.

Cordüan, G.: Production of tropane alkaloids in tissue cultures. - Planta med. **27** (Suppl.): 163, 1975.

Constable, F., Gamborg, D.L., Kurz, W.G.W., Steek, W.: Producton of secondary metabolites in plant cell cultures. - Planta med. **25**: 158-165, 1974.

Constabel, F.: Medicinal plant biotechnology. - Planta med. **66**: 421-425, 1990.

Gulati, A., Bharel, S., Abdin, M.Z., Jain, S.K., Srivastava, P.S.: *In vitro* micropropagation and flowering in *Artemisia annua*. - J. Plant Biochem. Biotechnol. **5**: 31-35, 1996a.

Gulati, A., Bharel, S., Srivastava, P.S., Abdin, M.Z., Jain, S.K.: Experimental studies in *Artemisia*, herbal remedy to malaria. - Fitoterapia **47**: 403-410, 1996b.

Khel, H., Kujundzić, N.: Effect of some hydroxamic acids on the growth of *Lepidium sativum* L. - Acta pharm. yugosl. **35**: 153-158, 1985.

Manot, A.R.: Secondary products through plant cell cultures. Problems and Prospects. - In: International Workshop on Tissue Culture and Biotechnology of Medical and Aromatic Plants. P. 17. Central Institute of Medicinal and Aromatic Plants, Lucknow 1987.

Murashige, T., Skoog, F.: A revised medium for rapid growth and bioassays with tobacco cultures. – Physiol. Plant. **15**: 473-497, 1962.

Purohit, M., Pande, D., Datta, A., Srivastava, P.S.: Enhanced xanthotoxin content in regenerating cultures of *Ammi majus* and micropropagation. - Planta med. **61**: 481-482, 1995a.

Purohit, M., Pande, D., Datta, A., Srivastava, P.S.: *In vitro* flowering and high xanthotoxin in *Ammi majus* L. – J. Plant Biochem. Biotechnol. **4**: 73-76, 1995b.

Reinert, J., Bajaj, Y.P.S.: Secondary products in tissue culture. - In: Butcher, D.N. (ed.): Plant Cell Tissue and Organ Culture. Pp. 668-693. Springer-Verlag, Berlin - Heidelberg - New York 1977.

Ramawat, K.G.: Secondary metabolites from plant tissue cultures. - In: Johri, B.M. (ed.): Botany in India: History and Progress. Vol II. Pp. 357-376. Oxford and IBH Publ. Co., New Delhi 1995.

Saba, Mughal, M.H., Ali, M., Iqbal, M., Srivastava, P.S.: A steryl ester from *Lepidium sativum*. - Phytochemistry **50**: 1375-1377, 1999.

Shimomura, K., Sudo, H., Saga, H., Kamada, H.: Shikonin production and secretion by hairy root cultures of *Lithospermum erythrorhizon*. - Plant Cell Rep. **10**: 282-285, 1991.

Smorodin, A.V., Smorodin, V.V., Bereznegovskaya, L.N.: [Investigation into growth conditions of *Datura innoxia* tissue suspension culture.] - Farmatsiya **3**: 29-34, 1979. [In Russ.]

Srivastava, P.S., Purohit, M., Pande, D., Dutta, A.: Phenotypic variation and alkaloid content in the androgenic plantlets of *Datura innoxia*. - Phytomorphology **43**: 209-216, 1993.

Srivastava, P.S., Pande, D.: *In vitro* propagation and conservation of medicinal plants. - In: Srivastava, P.S. (ed.): Plant Tissue Culture and Molecular Biology: Applications and Prospects. Pp. 254-281. Narosa Publ. House, New Delhi 1998.

Zenk, M.H.: Heavy metal detoxification in higher plants - a review. - Gene **179**: 21-30, 1996.