

Production of Plumbagin (5-hydroxy-2-methyl-1, 4-naphthoquinone) in Callus and Cell Suspension Cultures of *Plumbago indica* Linn.

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The relationship between growth and plumbagin (5-hydroxy-2-methyl-1, 4-naphthoquinone) formation in callus and cell suspension cultures of *Plumbago indica* was investigated. Semi-friable callus was induced on young stem and leaf segments in MS solid medium containing 1.5-2.5 mg l⁻¹ 2, 4-D plus 0.5-1.5 mg l⁻¹ KN. Cell suspension cultures were established from stem-derived semi-friable calli and cells released from aseptic leaf segments. Quantitative analysis of plumbagin in callus and cell cultures at different periods of growth revealed an increase in concentration of plumbagin with the increase in growth of cultures. Plumbagin level in both culture systems on unit dry wt basis was same throughout the growth of callus (0.05 mg/ g, dry wt) and cell cultures (0.028 mg/g, dry wt).

Keywords: *Plumbago indica*, callus, cell suspension cultures, plumbagin

Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) mainly occurs in the members of family *Plumbaginaceae* and *Droseraceae* (Kirtikar *et al*, 1975; Zenk *et al*, 1969). In *Plumbago indica* Linn. syn. *P. rosea* Linn (*Plumbaginaceae*), plumbagin is accumulated in the tuberous roots (0.9-1.0 %), and is endowed with significant anti-bacterial, anti-fertility and pesticidal properties (Dinda *et al*, 1997). There are only few reports on the production of plumbagin in tissue culture systems (Heble *et al*, 1974; Crouch *et al*, 1990). This paper describes the growth and plumbagin formation in callus and cell suspension cultures of *P. indica*.

Rooted cuttings (2-3 month old) of *P. indica*, grown in the pots, were used as the source of explants, and collected during June-August. For the induction of callus tissues, surface sterilized segments of internode (stem) (0.5-1.0cm) and leaf (1.0-1.5 cm²)

were placed on MS agar medium (Murashige & Skoog, 1962) containing 3% sucrose, auxins and cytokinins individually and in various combinations [2,4-D (2,4-dichloro phenoxyacetic acid), 0.5-3.0 mg l⁻¹; NAA (α -naphthalene acetic acid), 0.1-1.5 mg l⁻¹; IAA (indole 3-acetic acid), 0.1-1.5 mg l⁻¹; IBA (indole 3-butyric acid), 0.5-1.5 mg l⁻¹; KN (kinetin), 0.5-2.0 mg l⁻¹; BAP (benzylaminopurine), 0.5-3.0 mg l⁻¹]. The pH of the media was adjusted to 5.8 before adding 0.7% (w/v) agar (Hi Media Laboratories, Mumbai). The media were autoclaved at 15 psi for 18 min. The cultures were maintained at 26 $\pm$ 2°C under 12 hr. photoperiod (25-50 μ Em⁻²s⁻¹) provided with white fluorescent tube lights. The callus was maintained and multiplied by the same way by subculturing after 3-4 weeks. For growth studies, 1 g fresh wt (fw) callus was inoculated onto solid medium supplemented with various combinations and concentrations of auxins and cytokinins. The tissue was harvested at 10-day intervals over a 60-day long culture period and was dried and analyzed for plumbagin concentrations.

Cell suspension cultures were established from stem-derived semi-friable callus grown in 2.5mg l⁻¹ 2,4-D and 1.5 mg l⁻¹ KN, and from the cells directly released from aseptic leaf explants into the medium. Callus tissues (5g) were transferred to 250 ml Erlenmeyer flask containing 100 ml liquid medium supplemented with 2.5 mg l⁻¹ 2,4-D, 1.5 mg l⁻¹ KN, 1.5 mg l⁻¹ IBA and 1.0 mg l⁻¹ NAA, and shaken at 100 rpm. Cells in the medium (1.5 mg l⁻¹ IBA+1.0 mg l⁻¹ NAA) were collected through a nylon mesh and approximately 3 g cells transferred to medium for establishing cell suspension cultures.

Surface sterilized young leaf explants from field grown plants and leaf segments from aseptic shoot cultures were inoculated into plastic petri-dishes (6'x1') and 100 ml flasks containing liquid media supplemented with auxins at varied concentrations (0.5-1.5 mg l⁻¹ IBA or 0.5-1.5 mg l⁻¹ IBA + 0.1-1.0 mg l⁻¹ NAA and 0.5-1.5mg l⁻¹ IBA + 0.1-1.0 mg l⁻¹ IAA). The cultures were kept at both agitated (80 rpm) and non-agitated (still) conditions. The number of cells released into the media was calculated by manual counting of cells in 1 ml medium at ten different microscopic fields. After 3 weeks, cells

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released into the media, were allowed to sediment and these cells were transferred to 250 ml flasks containing liquid media with 1.5 mg l^{-1} IBA + 1.05 mg l^{-1} NAA for establishing cell suspension cultures. Cell suspension cultures raised through two sources were shaken at 100 rpm and regular subculturing was done at 5-day intervals over a 30-day long culture period. 10ml aliquots of cell suspension containing approximately 400-500 mg cells were transferred to media supplemented with various combinations of auxins ($0.5\text{--}1.5 \text{ mg l}^{-1}$ IBA or $0.5\text{--}1.5 \text{ mg l}^{-1}$ IBA + $0.1\text{--}1.0 \text{ mg l}^{-1}$ NAA, $0.5\text{--}1.5 \text{ mg l}^{-1}$ IBA + $0.1\text{--}1.0 \text{ mg l}^{-1}$ IAA) and studied the growth at 5-day intervals.

The cultured tissues (fw) were measured by carefully removing the adhered agar in case of callus and by filtering the leftover liquid medium through nylon mesh (20μ) under vacuum for suspension cultures. After determining the fresh wt, the tissues were dried at 60°C for 48 hr to measure the dry wt.

Plumbagin content in cultured tissues (callus and cells) had already been determined (Heble *et al*, 1974). 2 g of dried tissues was extracted at room temperature in chloroform for 8 hr or until all the pigments were leached out. The extract was filtered and concentrated under vacuum on a Buchii Rotavapour. The crude plumbagin obtained from callus/cell cultures was spotted onto 60 G Silica gel precoated TLC plates with known amount of authentic plumbagin (Sigma Co, USA). Benzene was used as a solvent at the lower face. The yellow spot corresponding to the authentic sample was scratched out and dissolved in CHCl_3 for quantitative measurements at 415 nm using a Shimadzu 2100 Spectrophotometer. HPLC of plumbagin was carried out (Crouch *et al*, 1990) on a Gilson 303 liquid chromatographic system with a Waters Bondapak C-18 column composed of solvent mixtures as methanol-acetic acid (80:20) buffered with trimethyl amine (pH 3.5). The flow rate was 1.0 ml min^{-1} and identification of the compound was based on a co-chromatography with authentic plumbagin and their retention time at 254 nm.

Stem (75%) and leaf (70%) explants, obtained as contamination free cultures, showed only marginal swelling in basal nutrient media. Whereas cultures grown in media supplemented with various hormones started callusing in 7 days of culture. Semi-friable callus formation was noticed on 86.6% of stem and 78.2% leaf explants cultured in media containing 2.5 mg l^{-1} 2,4-D, $+1.5 \text{ mg l}^{-1}$ KN and 1.5 mg l^{-1} 2,4-D + 0.5 mg l^{-1} KN respectively in a period of 3 weeks. In order

to establish stock callus cultures, explants with callus were subcultured to media with different hormones and a steady growth of callus was noticed in 5-6 weeks. For growth studies, 1 g (fw) callus was inoculated and noticed a slightly better growth in stem explant- derived callus. The highest growth of callus was obtained in media supplemented with 1.5 mg l^{-1} BAP + 1.0 mg l^{-1} NAA [leaf $8.68 \pm 0.14 \text{ g (fw)}$; stem $9.87 \pm 0.32 \text{ g (fw)}$] than in 2.5 mg l^{-1} 2,4-D + 1.5 mg l^{-1} KN [leaf, $8.4 \pm 0.05 \text{ g (fw)}$; stem, $9.23 \pm 0.09 \text{ g (fw)}$]. The results clearly indicate the requirement of NAA in preference to 2,4-D at concentrations less than that required for callus initiation. The results are in consonance with the earlier report (Yu & Chang, 1989) in *Angelica chinensis* that higher concentration of auxins was required for optimal callus induction and lower for subculture. Increase in concentration of BAP and NAA resulted in the loss of friability, formation of pigmentation and occasional differentiation of shoot buds as in *Hemidesmus indicus* (Sreekumar, 1998).

Analysis of callus growth showed a sigmoidal pattern of growth during the 60-day long culture passage. Biomass increase at 10-day intervals revealed that the maximum growth of tissues was in 30-40 days and grew to about eight times of initial inoculum (fw/dw) (Fig. 1). During the growth of callus tissues, plumbagin formation was maximum in 30-40 days ($0.066\text{--}0.099 \text{ mg/g dw}$). The concentration of plumbagin was closely followed the increase in fw of callus tissues. However, on dw basis, plumbagin content in calli was same ($0.04\text{--}0.05 \text{ mg/g}$) throughout the growth as reported in certain species (Cotton *et al*, 1991). In contrast, the concentration of poly galloylglucoses in callus cultures of *Rhus japonica* varied at different phases of growth (Tanguchi *et al*, 1997).

The release of stem-derived semi-friable callus and cells from aseptic leaf segments into the medium facilitated the establishment of suspension cultures. The latter source for establishing cell cultures was achieved by culture of leaf segments into media containing various concentrations and combinations of auxins. After 6-7 days, a slimy layer of parenchymatous cells appeared on the leaf lamina and gradually the cells released into the medium and continued to multiply. Leaf segments of field grown plants did not respond positively. Release of cells was prominent in cultures kept at shaken condition (80 rpm). Maximum number of cells released in 25 days by each explant in 1 ml medium was 58.56 ± 1.97 in

Table 1—Establishment of cell suspension cultures from leaf segments (*in vitro*- derived and field grown) cultured in MS liquid media containing different hormones. Number of cells released into the medium was recorded in 25 days of culture

PGRs mg l ⁻¹	Number of cells released/ ml nutrient medium			
	Leaf segments of <i>in vitro</i> -derived shoots		Leaf segments of field grown plants	
	Agitated	Still (non-agitated)	Agitated	Still (non-agitated)
0.5 IBA	18.12±0.63	3.83±0.32	0.3±0.014	-0-
1.0 IBA	23.29±1.46	2.30±0.17	0.26±0.02	-0-
1.5 IBA	51.32±2.12	21.65±3.65	1.78±0.09	-0-
1.0 IBA+0.5 IAA	58.56±1.97	18.3±2.40	2.9±0.07	-0-
1.5 IBA+1.0 IAA	14.6±0.68	2.96±0.65	2.0±0.12	-0-

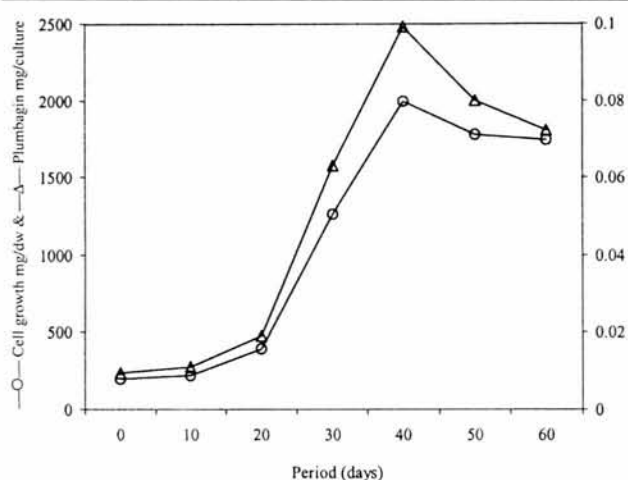


Fig. 1—Callus growth (○) and plumbagin (Δ) formation at different periods of culture in MS solid medium containing 1.5 mg/l IBA and 1.0 mg/l NAA. The results recorded at 10 day intervals

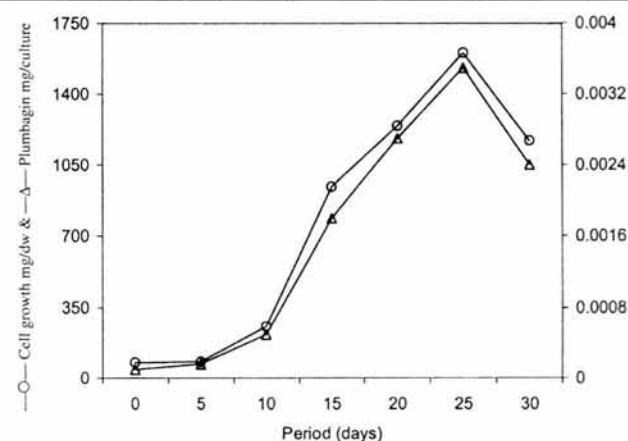


Fig. 2—Cell growth (○) in MS liquid medium containing 1.5 mg/l IBA and 1.0 mg/l NAA and formation of plumbagin (Δ) at different periods of culture. The results recorded at 5 day intervals.

medium supplemented with 1.0 mg l⁻¹ IBA and 0.5 mg l⁻¹ IAA. Number of cells released by the explants at still condition was less in the same hormonal combination (21.65±3.65) (Table 1). The cells were allowed to sediment, then harvested and subcultured to establish cell suspension cultures.

The biomass increase of cell cultures at 5-day intervals over a 30-day long culture period showed considerable improvement over callus cultures. The growth cycle of cell cultures derived from both sources was characterized by a short lag phase of 5 days when there was only marginal increase. A combination of 1.5 mg l⁻¹ IBA and 1.0 mg l⁻¹ NAA or 1.5 mg l⁻¹ NAA and 1.0 mg l⁻¹ BAP promoted maximum growth of cells followed by a combination of 1.5 mg l⁻¹ BAP and 1.0 mg l⁻¹ NAA. Between the latter two combinations, better growth of cells was obtained with 1.5 mg l⁻¹ IBA + 1.0 mg l⁻¹ NAA. After a short lag phase of 5 days, the culture entered into the

active exponential phase of growth between the 15th and 20th day attaining 18 to 20-fold increase, thereby declining on 25th day onwards. Plumbagin content in cells at different periods of culture was analyzed and an increase in concentration of plumbagin with increase in growth of cells was observed as in callus cultures (Fig. 2). The amount of plumbagin (0.018-0.027 mg/g dw) did not change throughout the culture period and yield of the compound was very poor. The authenticity of plumbagin was identified using TLC analysis (R_f value, 0.67) and HPLC profile (retention time, 5.60 min at 254 nm) as that of the standard.

In this study, callus and cell suspension cultures hold less importance since the concentration of plumbagin in culture systems is very less. The late log phase of callus and cell cultures accumulated plumbagin (0.0505 mg and 0.0268 mg/g dw), which is very less as compared to plumbagin (9-10.0 mg/g dw) from the roots of 16-20 month old field grown cultivated plants. At the same time, the use of aseptic

shoot-derived leaf segments for raising cell suspension cultures through the direct release of cells into the medium deserves special attention because this method enables bypassing the time consuming steps of callus development.

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