

# Thidiazuron-induced shoot organogenesis and production of hepatoprotective lignan phyllanthin and hypophyllanthin in *Phyllanthus amarus*

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**Abstract** Callus cultures from nodal and leaf explants of *Phyllanthus amarus* were established on Murashige and Skoog (MS) medium with various combinations of auxins and cytokinins. The leaf-derived callus induced on 2.26  $\mu\text{M}$  2,4-dichlorophenoxyacetic acid (2, 4-D) + 2.32  $\mu\text{M}$  Kinetin (Kin) upon transfer to medium containing thidiazuron (TDZ) exhibited higher shoot regeneration (32.4  $\pm$  1.3 shoots per culture). Four-week-old shoots rooted readily on 1.5  $\mu\text{M}$  Indol acetic acid (IAA)-containing medium and were successfully acclimatized with 98% survival. The lignans, Phyllanthin (PH) and Hypophyllanthin (HPH), of leaf extracts from naturally grown plants were identified by using TLC, HPLC and H1-NMR. The PH and HPH production in the regenerated shoots was compared to their production in callus cultures, plants under field conditions and in naturally grown plants. The regenerated shoots on MS + 2.27  $\mu\text{M}$  TDZ produced about two times higher PH and HPH than the leaves of naturally grown plant. The present study provides a useful system for further studies on in vitro morphogenesis, elicitor-assisted production of PH and HPH and *A. rhizogenes*-mediated genetic transformation in *Phyllanthus amarus*.

**Keywords** *Phyllanthus amarus* · Phyllanthin · Hypophyllanthin · Callus · Regeneration

## Abbreviations

BA	6-benzyladenine
Kin	Kinetin 6-furfurylaminopurin
TDZ	Thidiazuron(1-phenyl-3-(1,2,3-thiadiazol-5-yl) urea)
2,4-D	2,4-dichlorophenoxyacetic acid
NAA	$\alpha$ -naphthalene acetic acid
IAA	Indol acetic acid
HPLC	High performance liquid chromatography
TLC	Thin-layer chromatography
NMR	Nuclear Magnetic resonance

## Introduction

*Phyllanthus amarus* Shum and Thonn (Euphorbiaceae) is an annual herb widely distributed in the tropical and subtropical regions of India and Central and South American countries (Ghanti et al. 2004). Due to its numerous pharmaceutical applications, this herb is used in traditional folk medicines in India (Unander et al. 1995; Sharma et al. 2001). It is often used for the treatment of fever, jaundice, ascites, hemorrhoid, frequent menstruation, skin ulcer and diabetes (Rai and Mehrotra 2007). Apart from these medicinal uses, several reports showed that the whole plant possesses hypoglycemic, antibacterial, antifungal and antiviral (Sharma et al. 2001), antinociceptive (Santos et al. 2000), antitumor (Islam et al. 2008), antimutagenic (Kumar and Kuttan 2005), and anti-inflammatory (Kierner et al. 2003) properties. It is employed in an Ayurvedic

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preparation used as a health promotive and disease preventive tonic (Govindarajan et al. 2007).

This herb has strong activity against hepatitis B virus (HBV) (Shim et al. 2000) and C virus (HCV) (Bhattacharyya et al. 2003). The hepatoprotective active ingredients were shown to be phyllanthin (PH) and (HPH) hypophyllanthin (Shim et al. 2000). The chemical synthesis of PH and HPH is difficult due to their complex structure.

In nature, *P. amarus* appears along with other herbs after the first showers of rain and disappears on completion of the monsoon. The rate of propagation through seed is unreliable due to poor seed germination and seed heterothermy (Singh et al. 2006). As the plant parts are smaller in size, the resultant biomass is insufficient. Overexploitation of this species by pharmaceutical companies, firms preparing Ayurvedic medicines and grazing animals has resulted in rapid depletion of its natural stock. Considering the ever-increasing demand for this plant, it is necessary to develop alternate propagation strategies. There are no well-defined methods for organized cultivation of this plant. Therefore, the plants growing in the uncultivated areas have to be used which also limits the supply of the quality material. During the last few decades, organ, tissue and callus culture have been reported as an alternative method for production of biomass used for extraction of active metabolites (Rao and Ravishankar 2002).

Despite having immense medicinal importance, *P. amarus* has so far received inadequate attention. In vitro propagation techniques have been reported for *P. carolinensis* (Catapan et al. 2000), *P. urinaria* (Catapan et al. 2002), and *P. stipulatus* (Catapan et al. 2001). Synthetic seeds of alginate encapsulated shoot tips were reported in *P. amarus* (Singh et al. 2006). Shoot tip, nodal and internodal segments were used for in vitro plantlet regeneration in *P. amarus* (Bhattacharyya and Bhattacharyya 2001; Ghanti et al. 2004). Genetic transformation protocol has been recently described for *P. amarus* (Banerjee and Chattopadhyay 2009; Abhyankar et al. 2010). However, there are no reports on shoot organogenesis in relation to accumulation of phyllanthin and hypophyllanthin. Therefore, the aim of the present investigation was to establish a protocol for efficient propagation through callus culture, to study the organogenic response mediated through different plant growth regulators and biosynthesis of PH and HPH.

## Materials and methods

### Plant material and surface sterilization

The plants of *P. amarus* were collected from naturally grown collections found on the campus of University of Pune during the months of June and August in the years

2005, 2006 and 2007. A voucher specimen (BSI/WRC/Tech/2010/NITKPA-1) was deposited in the herbarium of the regional office of the Botanical Survey of India, Pune 411 001. The nodal and leaflet explants (10 mm) were randomly selected from healthy mother plants (height about 20–30 cm). The explants were thoroughly washed with tap water and surface sterilized with 0.1% (W/V) mercuric chloride for 5 min, followed by five washes with sterile distilled water.

### Culture conditions and establishment of callus culture

Surface sterilized explants were transferred to MS (Murashige and Skoog 1962) medium supplemented with cytokinins (BA, Kin and TDZ) (0–22  $\mu\text{M}$ ) and auxins (IAA, NAA and 2, 4-D) (0–22  $\mu\text{M}$ ), singly and in combinations. The media were fortified with 3% (W/V) sucrose, 0.8% agar (W/V), and pH was adjusted to 5.8. The developed calluses were separated from the initial explants and healthy masses subsequently sub-cultured at about 4 weeks interval. Fresh callus (1 g) was used as inoculum. The calluses were harvested after 4 weeks and dried in an oven at 60°C until constant weight was obtained. The calluses obtained on different media were analyzed for PH and HPH. Cultures were incubated at  $25 \pm 2^\circ\text{C}$  under 8 h photoperiod, with light of  $30 \mu\text{mol m}^{-2} \text{s}^{-1}$  intensity from cool fluorescent tubes (40 W; Philips India) for a period of 4 weeks.

### Shoot and root formation in the callus

To study the influence of different concentrations and types of cytokinins and auxins on shoot and root regeneration, MS basal medium supplemented with BA (00–22.19  $\mu\text{M}$ ), Kin (2.32–23.23  $\mu\text{M}$ ), TDZ (00–40  $\mu\text{M}$ ), IAA (0.57–28.54  $\mu\text{M}$ ), NAA (0.54–26.85  $\mu\text{M}$ ) and 2, 4-D (0.45–22.62  $\mu\text{M}$ ) singly and in combinations was used. Pieces of about 1 g calluses were transferred onto medium for shoot and root regeneration. Cultures were incubated at  $25 \pm 2^\circ\text{C}$ , under 8 h photoperiod, with light intensity of  $30 \mu\text{mol m}^{-2} \text{s}^{-1}$  provided by cool fluorescent tubes (Philips India) for a period of 4 weeks.

### Rooting and transplantation

After 4 weeks of incubation, the regenerated shoots (2–3 cm) were separated from clumps and harvested for rooting in MS medium supplemented with IAA or NAA (00–22  $\mu\text{M}$ ). The frequency of rooting and average number of roots per shoot were determined and recorded after 28 days of culture. The plantlets were removed and washed

with water to remove traces of agar. The young plants were transplanted into earthen pots containing soil and sand mixture (2:1) and kept in glass house for 2 weeks (light  $60 \mu\text{mol m}^{-2} \text{s}^{-1}$ , humidity 80–90% and temperature  $25 \pm 4^\circ\text{C}$ ) and then gradually exposed to ambient humidity.

#### Isolation of PH and HPH

PH and HPH were isolated as per the method of Row and Srinivasulu (1966). The oven-dried leaves (300 g) were mixed with lime (90 g) and water (90 ml) and allowed to dry overnight before extraction with petroleum ether (4 syphonings). The bright yellow semi-solid residue (7 g), obtained after removal of the solvent by distillation, was boiled with ethanol (100 ml) for 10 min, cooled to room temperature, and then filtered. The filtrate after evaporation gave pale yellowish residue (4–5 g). The residue was boiled with petroleum ether and left until separation of yellow oil was complete. On cooling, it gave a mixture of yellow and white powder (2–3 g). Part of the residue (1 g) was layered over Si gel (60–120 mesh LR 40 g) column and was eluted with hexane and mixture of hexane and ethyl acetate. The polarity of the eluting solvent was increased gradually and fractions of 50 ml volume were collected. At 20% polarity, fraction number 75–84 gave mixture of PH and HPH after removal of the solvent, which was confirmed on TLC. These fractions were pooled and evaporated to yellow residue (50 mg). Part of this residue (10 mg) was further purified on preparative HPLC by using MeOH: water (65:35 v/v) as mobile phase.

#### NMR analysis

$^1\text{H}$  (proton) spectra were recorded in  $\text{CDCl}_3$  using a 300 MHz Varian Mercury (Bruker, Dubendorf, Switzerland) equipped with a QNP probehead. Chemical shifts ( $\delta$ ) were reported in parts per million (ppm) related to tetramethylsilane as internal standard and coupling constant ( $j$ ) was given in Hertz. The spectra were in complete agreement with those reported in literature.

#### Preparation of sample extract

About 0.5 g of powdered callus, natural material (fruit, leaves, and stem) and part of in vitro raised shoots (fruit, leaves, and stem) of *P. amarus* were extracted in 5 ml petroleum ether in an ultrasonic bath for 30 min. After centrifugation, the supernatant was evaporated to dryness. The residue was dissolved in 100  $\mu\text{l}$  HPLC grade methanol, 20  $\mu\text{l}$  of which was submitted to HPLC analysis.

#### Estimation of PH and HPH

The isocratic HPLC system (DIONEX) comprised P-680 solvent delivery pump, C18 ( $250 \times 4.6 \text{ mm}$ ,  $5 \mu\text{m}$ ) column and UV detector (170 U). Mobile phase used was methanol–water (65:35 v/v) with a flow rate 1.0 ml/min at  $25^\circ\text{C}$ . Twenty  $\mu\text{l}$  sample was injected and scanned at 254 nm. One mg powder of PH and HPH was dissolved in 1 ml HPLC grade methanol. The peaks corresponding to these lignans were confirmed by spiking the sample extract along with standard. The PH and HPH in the samples were quantified by comparing the peak areas with those of the standard PH and HPH.

#### Statistical analysis

All the experiments were set up in completely randomized design. Each treatment consisted of at least 21 replicates. All the treatments were repeated thrice. Mean values were compared using Duncan's Multiple Range Test (DMRT) at 5% probability (Duncan 1955).

## Result and discussion

#### Callus induction and growth of callus

Leaf and nodal explants of *P. amarus* grown on MS medium supplemented with BA (2.22  $\mu\text{M}$ –22.19  $\mu\text{M}$ ) or Kin (2.32  $\mu\text{M}$ –23.23  $\mu\text{M}$ ) or TDZ (2.27  $\mu\text{M}$ –40  $\mu\text{M}$ ) formed calluses from the cut ends within 2 weeks of culture, and at the end of the fourth week, the entire surface of explant was covered with callus. The amount, texture, color and morphogenic response and PH and HPH contents of these calluses varied with the type and concentration of cytokinin (Tables 1, 2).

Extensive proliferation and growth of callus (dry weight basis) in both the explants were observed with significant differences on medium supplemented with lower concentrations of BA (2.22  $\mu\text{M}$ ) or TDZ (2.27  $\mu\text{M}$ ) or Kin (2.32  $\mu\text{M}$ ) (Tables 1, 2). However, the leaf-derived callus showed significantly higher growth (295 mg DW per culture) compared to the node-derived callus (136 mg DW per culture) on 2.22  $\mu\text{M}$  BA-containing medium (Table 1). The callus was dark green, nodular and hard on medium with BA and TDZ, while loose and pale green on medium with Kin. BA appears to be superior compared to Kin and TDZ for callus induction in *P. amarus*. The use of MS medium containing BA was also shown to be effective for callus induction from nodal explant in *P. stipulatus* (Catapan et al. 2001).

It is well known that 2, 4-D promotes cell division and is mostly employed for callus induction in tissue culture

**Table 1** Effect of cytokinin (BA, Kin) on callus formation and accumulation of phyllanthin and hypophyllanthin in callus culture of *P. amarus*

Cytokinin $\mu\text{M}$	Callus derived from leaf explant			Morphology of callus	Callus derived from nodal explant		
	Dry wt. mg	Phyllanthin $\mu\text{g/g DW}$	Hypophyllanthin $\mu\text{g/g DW}$		Dry wt. mg	Phyllanthin $\mu\text{g/g DW}$	Hypophyllanthin $\mu\text{g/g DW}$
BA							
00	–	–	–				
2.22	295 $\pm$ 1.4 <sup>a</sup>	42.1 $\pm$ 1.2 <sup>a</sup>	26.9 $\pm$ 1.5 <sup>a</sup>	Green, compact	136 $\pm$ 1.5 <sup>a</sup>	32.1 $\pm$ 0.2 <sup>a</sup>	16.9 $\pm$ 0.5 <sup>a</sup>
4.44	144 $\pm$ 2.2 <sup>bc</sup>	32.2 $\pm$ 0.4 <sup>b</sup>	24.3 $\pm$ 0.3 <sup>a</sup>	Green, compact	129 $\pm$ 1.3 <sup>a</sup>	29.1 $\pm$ 0.1 <sup>b</sup>	12.2 $\pm$ 0.7 <sup>b</sup>
8.87	98 $\pm$ 2.1 <sup>d</sup>	26.1 $\pm$ 0.5 <sup>d</sup>	13.2 $\pm$ 0.5 <sup>c</sup>	White, soft	103 $\pm$ 1.2 <sup>b</sup>	21.5 $\pm$ 0.4 <sup>c</sup>	10.2 $\pm$ 0.4 <sup>cd</sup>
15.0	76 $\pm$ 1.7 <sup>d</sup>	28.2 $\pm$ 0.2 <sup>c</sup>	12.2 $\pm$ 0.8 <sup>cd</sup>	White, soft	56 $\pm$ 1.2 <sup>d</sup>	11.5 $\pm$ 0.8 <sup>e</sup>	9.5 $\pm$ 0.4 <sup>cd</sup>
22.19	49 $\pm$ 1.3 <sup>e</sup>	10.3 $\pm$ 0.4 <sup>g</sup>	4.6 $\pm$ 0.1 <sup>f</sup>	Brown, loose	32 $\pm$ 3.1 <sup>e</sup>	10.4 $\pm$ 0.6 <sup>e</sup>	6.7 $\pm$ 0.3 <sup>e</sup>
Kin							
2.32	168 $\pm$ 3.2 <sup>b</sup>	29.2 $\pm$ 0.5 <sup>c</sup>	16.2 $\pm$ 0.2 <sup>b</sup>	Pale green, loose	97 $\pm$ 2.1 <sup>b</sup>	11.2 $\pm$ 0.1 <sup>e</sup>	12.1 $\pm$ 0.1 <sup>b</sup>
4.65	134 $\pm$ 2.4 <sup>c</sup>	28.2 $\pm$ 0.3 <sup>c</sup>	12.5 $\pm$ 0.4 <sup>c</sup>	Pale green, loose	78 $\pm$ 1.5 <sup>c</sup>	19.0 $\pm$ 0.5 <sup>d</sup>	10.5 $\pm$ 0.6 <sup>bc</sup>
9.29	129 $\pm$ 4.1 <sup>c</sup>	12.6 $\pm$ 0.1 <sup>f</sup>	10.5 $\pm$ 0.5 <sup>d</sup>	Pale green, loose	64 $\pm$ 1.2 <sup>cd</sup>	10.8 $\pm$ 0.1 <sup>e</sup>	8.6 $\pm$ 0.9 <sup>d</sup>
15.0	94 $\pm$ 2.3 <sup>d</sup>	18.3 $\pm$ 0.4 <sup>e</sup>	8.2 $\pm$ 0.7 <sup>e</sup>	White, soft	59 $\pm$ 3.2 <sup>d</sup>	7.8 $\pm$ 0.2 <sup>f</sup>	6.4 $\pm$ 0.01 <sup>e</sup>
23.23	37 $\pm$ 1.9 <sup>e</sup>	10.2 $\pm$ 0.8 <sup>g</sup>	7.5 $\pm$ 0.3 <sup>e</sup>	White, soft	28 $\pm$ 2.1 <sup>e</sup>	6.3 $\pm$ 0.2 <sup>f</sup>	5.7 $\pm$ 0.02 <sup>e</sup>

The values represent the mean  $\pm$  SE calculated from three independent experiments, each based on a minimum 21 replicates. Values followed by same superscript letter were not significantly different at 5% level (DMRT)

**Table 2** Influence of TDZ on callus formation and biosynthesis of phyllanthin and hypophyllanthin in callus culture of *P. amarus*

Cytokinin $\mu\text{M}$	Callus derived from leaf explant			Morphology of callus	Callus derived from nodal explant		
	Dry wt. mg	Phyllanthin $\mu\text{g/g DW}$	Hypophyllanthin $\mu\text{g/g DW}$		Dry wt. mg	Phyllanthin $\mu\text{g/g DW}$	Hypophyllanthin $\mu\text{g/g DW}$
TDZ							
00	–	–	–	–	–	–	–
0.45	148 $\pm$ 1.3 <sup>b</sup>	38.2 $\pm$ 1.2 <sup>a</sup>	25.2 $\pm$ 0.3 <sup>b</sup>	Green, compact	139 $\pm$ 1.2 <sup>bc</sup>	25.2 $\pm$ 1.4 <sup>a</sup>	12.3 $\pm$ 0.1 <sup>c</sup>
2.27	183 $\pm$ 1.7 <sup>a</sup>	23.5 $\pm$ 2.5 <sup>b</sup>	29.6 $\pm$ 0.2 <sup>a</sup>	Green, compact	166 $\pm$ 1.5 <sup>a</sup>	22.2 $\pm$ 0.2 <sup>b</sup>	16.5 $\pm$ 0.3 <sup>a</sup>
4.54	172 $\pm$ 1.9 <sup>ab</sup>	20.0 $\pm$ 1.3 <sup>b</sup>	21.2 $\pm$ 0.1 <sup>c</sup>	Green, compact	157 $\pm$ 1.3 <sup>ab</sup>	19.6 $\pm$ 0.5 <sup>cd</sup>	14.2 $\pm$ 0.2 <sup>b</sup>
9.08	136 $\pm$ 2.2 <sup>bc</sup>	12.5 $\pm$ 4.1 <sup>c</sup>	19.8 $\pm$ 0.3 <sup>c</sup>	Light green soft	135 $\pm$ 2.1 <sup>c</sup>	21.3 $\pm$ 0.4 <sup>b</sup>	11.2 $\pm$ 0.1 <sup>cd</sup>
22.71	113 $\pm$ 2.5 <sup>c</sup>	14.2 $\pm$ 1.6 <sup>c</sup>	16.4 $\pm$ 0.4 <sup>d</sup>	Light green soft	112 $\pm$ 2.4 <sup>d</sup>	18.2 $\pm$ 0.9 <sup>d</sup>	10.2 $\pm$ 0.4 <sup>d</sup>
40	96 $\pm$ 2.1 <sup>c</sup>	16.5 $\pm$ 2.1 <sup>c</sup>	10.6 $\pm$ 0.2 <sup>e</sup>	Light green soft	–	–	–

The values represent the mean  $\pm$  SE calculated from three independent experiments, each based on a minimum 21 replicates. Values followed by same superscript letter were not significantly different at 5% level (DMRT)

studies (Constabel 1987). In the present investigation, among the auxins used, maximum growth of callus (199 mg DW per culture) in leaf explants was recorded on medium containing 4.52  $\mu\text{M}$  2, 4-D. Substantial callus formation was also observed with 10.74  $\mu\text{M}$  NAA and 11.42  $\mu\text{M}$  IAA. Similar trend of callus formation was also recorded in nodal explants on equimolar concentrations of 2, 4-D, NAA and IAA (Table 3) with significant differences. Concurrent results were reported for establishment of callus in *P. urinaria*, *P. abnormis* and *P. carolinensis* (Catapan et al. 2000, 2002). Therefore, 2, 4-D seems to be superior over other auxins when used singly for callus formation in *Phyllanthus* sp.

In the present investigation, the callus produced on media supplemented with IAA and NAA was off-white and

friable and produced numerous roots (1–12 roots per culture) in the third and fourth weeks of culture. However, callus produced on 2,4-D-fortified media was yellowish and loose without any root formation (Table 3). In an earlier report in *P. stipulatus*, root formation in the callus was recorded on media fortified with 1.25–5  $\mu\text{M}$  NAA (Catapan 1999). Use of cytokinins in conjunction with auxins facilitated only callus formation (Table 4). From a total of more than 240 combinations of auxins and cytokinins tested at various concentrations (0.45–40  $\mu\text{M}$ ), significant callus proliferation from leaf explant was observed with 4.65  $\mu\text{M}$  Kin + 2.15  $\mu\text{M}$  NAA, while from nodal explant, it was observed with 4.44  $\mu\text{M}$  BA + 0.54  $\mu\text{M}$  NAA (Table 4). Therefore, growth regulator requirement for callus induction appeared to vary with the source of explant.

**Table 3** Effect of Auxins (IAA, NAA and 2, 4-D) on callus induction and biosynthesis of phyllanthin and hypophyllanthin in *P. amarus*

Auxins μM	Callus derived from leaf explant				Callus derived from Nodal explant			
	Dry wt. mg	No. of roots per culture	Phyllanthin μg/g DW	Hypophyllanthin μg/g DW	Dry wt. mg	No. of roots per culture	Phyllanthin μg/g DW	Hypophyllanthin μg/g DW
NAA								
00	–	–	–	–	–	–	–	–
0.54	104 ± 2.2 <sup>k</sup>	1.2 ± 0.02 <sup>a*</sup>	0.75 ± 0.01 <sup>b</sup>	0.36 ± 0.02 <sup>b</sup>	118 ± 5.1 <sup>gh</sup>	8.0 ± 0.2 <sup>d*</sup>	0.45 ± 0.05 <sup>c</sup>	0.12 ± 0.02 <sup>ab</sup>
2.15	107 ± 1.4 <sup>j</sup>	1.4 ± 0.02 <sup>a*</sup>	0.84 ± 0.06 <sup>a</sup>	0.38 ± 0.01 <sup>a</sup>	126 ± 4.3 <sup>fgh</sup>	8.9 ± 0.2 <sup>c*</sup>	0.35 ± 0.01 <sup>de</sup>	0.10 ± 0.04 <sup>cd</sup>
5.37	161 ± 1.4 <sup>c</sup>	1.3 ± 0.01 <sup>a*</sup>	0.62 ± 0.03 <sup>c</sup>	0.24 ± 0.03 <sup>e</sup>	138 ± 1.6 <sup>def</sup>	9.7 ± 0.1 <sup>b*</sup>	0.48 ± 0.03 <sup>b</sup>	0.13 ± 0.02 <sup>ab</sup>
10.74	184 ± 2.5 <sup>c</sup>	1.2 ± 0.01 <sup>a*</sup>	0.52 ± 0.06 <sup>d</sup>	0.19 ± 0.02 <sup>g</sup>	149 ± 2.4 <sup>bcd</sup>	9.5 ± 0.1 <sup>b*</sup>	0.36 ± 0.05 <sup>d</sup>	0.09 ± 0.01 <sup>de</sup>
26.85	78 ± 1.9 <sup>l</sup>	1.2 ± 0.05 <sup>a*</sup>	0.32 ± 0.02 <sup>f</sup>	0.16 ± 0.04 <sup>h</sup>	109 ± 2.2 <sup>h</sup>	7.4 ± .21 <sup>e*</sup>	0.26 ± 0.06 <sup>g</sup>	0.07 ± 0.04 <sup>f</sup>
IAA								
0.57	127 ± 3.4 <sup>i</sup>	0.9 ± 0.05 <sup>a*</sup>	0.65 ± 0.01 <sup>c</sup>	0.28 ± 0.01 <sup>d</sup>	108 ± 6.3 <sup>h</sup>	5.1 ± 0.2 <sup>f*</sup>	0.34 ± 0.02 <sup>e</sup>	0.10 ± 0.02 <sup>cd</sup>
2.85	143 ± 2.5 <sup>g</sup>	1.2 ± 0.11 <sup>a*</sup>	0.54 ± 0.02 <sup>d</sup>	0.32 ± 0.05 <sup>c</sup>	124 ± 3.5 <sup>fgh</sup>	7.8 ± 0.1 <sup>de*</sup>	0.45 ± 0.03 <sup>c</sup>	0.12 ± 0.04 <sup>ab</sup>
5.71	148 ± 2.7 <sup>f</sup>	1.3 ± 0.02 <sup>a*</sup>	0.68 ± 0.04 <sup>c</sup>	0.36 ± 0.02 <sup>b</sup>	132 ± 1.4 <sup>efg</sup>	11.6 ± 0.3 <sup>a*</sup>	0.56 ± 0.01 <sup>a</sup>	0.13 ± 0.04 <sup>a</sup>
11.42	177 ± 2.5 <sup>d</sup>	1.2 ± 0.02 <sup>a*</sup>	0.46 ± 0.02 <sup>e</sup>	0.23 ± 0.01 <sup>ef</sup>	166 ± 3.9 <sup>ab</sup>	4.2 ± 0.2 <sup>g*</sup>	0.32 ± 0.05 <sup>f</sup>	0.11 ± 0.02 <sup>bc</sup>
28.54	126 ± 3.1 <sup>i</sup>	1.1 ± 0.02 <sup>a*</sup>	0.32 ± 0.09 <sup>f</sup>	0.24 ± 0.06 <sup>e</sup>	132 ± 1.4 <sup>defg</sup>	3.9 ± 0.8 <sup>g*</sup>	0.23 ± 0.01 <sup>h</sup>	0.11 ± 0.01 <sup>bc</sup>
2,4-D								
0.45	127 ± 2.6 <sup>i</sup>	C	0.23 ± 0.03 <sup>g</sup>	0.22 ± 0.03 <sup>f</sup>	164 ± 1.7 <sup>abc</sup>	EC	0.13 ± 0.02 <sup>ij</sup>	0.08 ± 0.02 <sup>ef</sup>
2.26	138 ± 1.9 <sup>h</sup>	C	0.32 ± 0.01 <sup>f</sup>	0.16 ± 0.05 <sup>h</sup>	147 ± 1.4 <sup>cde</sup>	EC	0.13 ± 0.05 <sup>ij</sup>	0.04 ± 0.02 <sup>g</sup>
4.52	199 ± 4.3 <sup>a</sup>	EC	0.26 ± 0.02 <sup>fg</sup>	0.10 ± 0.02 <sup>i</sup>	172 ± 1.8 <sup>a</sup>	EC	0.16 ± 0.06 <sup>h</sup>	0.10 ± 0.03 <sup>cd</sup>
9.05	186 ± 2.3 <sup>b</sup>	C	0.15 ± 0.03 <sup>h</sup>	0.09 ± 0.01 <sup>i</sup>	133 ± 2.2 <sup>defg</sup>	C	0.14 ± 0.02 <sup>i</sup>	0.09 ± 0.01 <sup>de</sup>
22.62	105 ± 1.8 <sup>k</sup>	C	0.10 ± 0.02 <sup>h</sup>	0.09 ± 0.02 <sup>i</sup>	139 ± 2.2 <sup>def</sup>	C	0.12 ± 0.07 <sup>j</sup>	0.04 ± 0.04 <sup>g</sup>

The values represent the mean ± SE calculated from three independent experiments, each based on a minimum 21 replicates. Values followed by same superscript letter were not significantly different at 5% level (DMRT)

EC Explant produced extensive callus, C explant produced callus, \*callus interspersed root formation

**Table 4** Influence of cytokinins and auxins in combination on callus growth and accumulation of phyllanthin and hypophyllanthin in callus of *P. amarus*

Growth regulators μM	Callus derived from leaf explant			Callus derived from nodal explant		
	Dry wt. mg	Phyllanthin μg/g DW	Hypophyllanthin μg/g DW	Dry wt. mg	Phyllanthin μg/g DW	Hypophyllanthin μg/g DW
4.44 μM BA + 0.54 μM NAA	183 ± 1.4 <sup>ab</sup>	17.2 ± 0.2 <sup>a</sup>	7.6 ± 0.1 <sup>a</sup>	128 ± 3.3 <sup>a</sup>	16.7 ± 0.2 <sup>a</sup>	6.8 ± 0.4 <sup>a</sup>
4.65 μM Kin + 2.15 μM NAA	193 ± 1.7 <sup>a</sup>	7.5 ± 0.2 <sup>d</sup>	1.9 ± 0.7 <sup>c</sup>	119 ± 1.4 <sup>ab</sup>	4.2 ± 0.4 <sup>d</sup>	0.7 ± 0.2 <sup>e</sup>
4.54 μM TDZ + 2.85 μM NAA	172 ± 3.6 <sup>b</sup>	16.8 ± 0.2 <sup>a</sup>	4.5 ± 0.03 <sup>b</sup>	113 ± 3.2 <sup>abc</sup>	10.8 ± 0.4 <sup>b</sup>	3.2 ± 0.3 <sup>b</sup>
8.87 μM BA + 0.54 μM IAA	152 ± 2.6 <sup>c</sup>	13.1 ± 0.1 <sup>b</sup>	7.6 ± 0.4 <sup>a</sup>	98 ± 4.2 <sup>c</sup>	6.5 ± 0.5 <sup>c</sup>	1.9 ± 0.1 <sup>c</sup>
2.32 μM Kin + 5.71 μM IAA	189 ± 1.3 <sup>b</sup>	12.3 ± 0.4 <sup>b</sup>	3.6 ± 0.2 <sup>bc</sup>	106 ± 2.4 <sup>bc</sup>	9.7 ± 0.5 <sup>b</sup>	1.5 ± 0.2 <sup>cd</sup>
2.32 μM Kin + 2.26 μM 2,4-D	181 ± 2.1 <sup>ab</sup>	9.7 ± 0.1 <sup>c</sup>	2.1 ± 0.1 <sup>c</sup>	124 ± 1.7 <sup>ab</sup>	4.6 ± 0.3 <sup>d</sup>	0.9 ± 0.2 <sup>cd</sup>

The values represent the mean ± SE calculated from three independent experiments, each based on a minimum 21 replicates. Values followed by same superscript letter were not significantly different at 5% level (DMRT)

Though substantial callus growth was also obtained with other combinations of growth regulators (Tables 1, 2, 3 and 4), texture and color of the callus and rhizogenesis response depended on auxin and cytokinin. The higher proportion of IAA or NAA turned the callus friable and whitish in color and showed rhizogenesis at the end of the fourth week

(Table 3). On the other hand, a lower proportion of auxin made the callus dark or pale green and nodular. Both leaf and nodal explant-derived calluses with regular sub-culturing to fresh medium after every 28 days were kept in an actively growing condition over 2 years. These results reveal that, in *P. amarus*, the best callogenic response can

be obtained in the presence of 2.22  $\mu\text{M}$  BA in the MS medium (Table 1; Fig. 1a).

#### Accumulation of PH and HPH in callus

The structures of PH and HPH isolated from the leaves of *P. amarus* were confirmed by comparing their NMR spectra with those reported previously (Somanabandhu et al. 1993). These PH and HPH extracted and separated were used as standard compounds. Considering the retention time with the standards, the peak with retention time of  $14.6 \pm 0.8$  min and  $12.9 \pm 0.5$  min was assigned to PH and HPH, respectively.

The accumulation of PH and HPH was higher in the callus obtained on low concentrations of cytokinins (Table 1). Higher concentration of cytokinins in the medium was inhibitory for growth of callus as well as accumulation of PH and HPH. The levels of PH and HPH were observed to be higher in the leaf-derived callus raised on BA-containing medium, followed by TDZ- and Kin-containing media. The callus derived from leaf explant showed significantly higher levels of PH and HPH as compared to node derived callus on BA containing medium (Table 1).

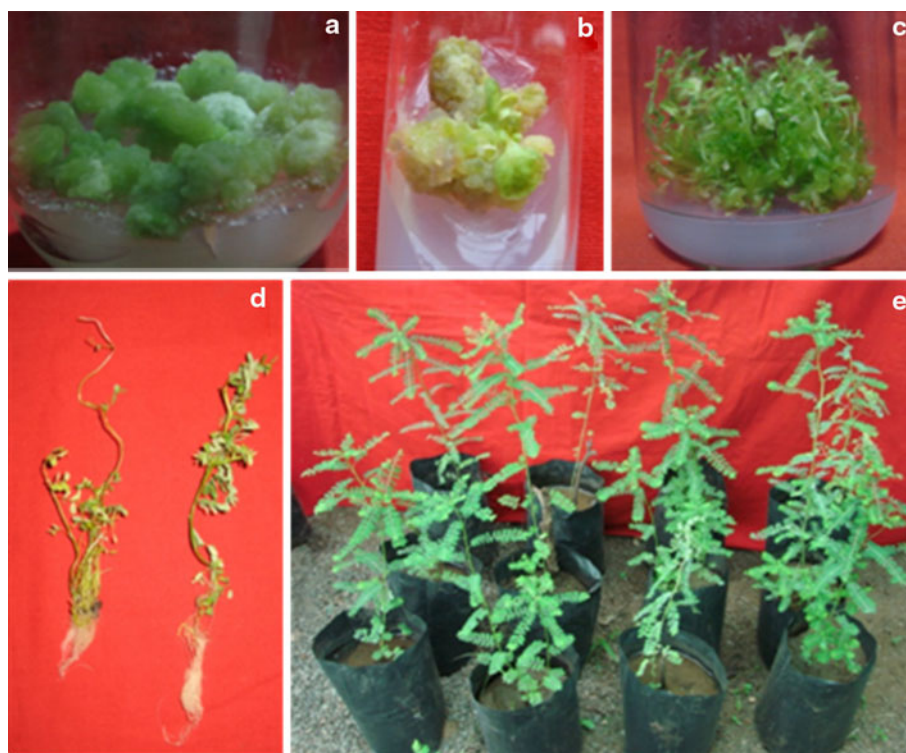
The calluses raised on MS medium incorporated with NAA showed higher levels of PH and HPH compared to IAA (Table 3), whereas lower levels were observed in the calluses obtained on 2,4-D-containing medium. These results are in conformity to earlier reports (Ramawat and

Merrilion 1999), where auxins, particularly 2,4-D, was shown to promote de-differentiation and primary metabolism and suppress secondary metabolism. However, incorporation of auxins in the MS medium resulted in inverse relationship between accumulation of PH and HPH in callus and growth of callus. Possibly, the diversion of carbon flux from the secondary metabolic pathway, low activity of key enzymes, lack of appropriate storage sites or transport mechanism or unregulated catabolism of the product are responsible for such effects (Dicosmo and Misawa 1996).

Incorporation of auxins together with cytokinins reduced the accumulation of PH and HPH in the calluses derived from leaf as well as nodal explant (Table 4). The most potent inhibitor was 2, 4-D, followed by IAA and NAA. Among the explants, callus derived from leaf explant contained higher levels of PH and HPH compared to that of callus derived from nodal explant (Table 4). This trend remained the same irrespective of type and concentration of growth regulator. However, the dry weight of the callus and accumulation of PH and HPH in the callus produced on MS medium containing 2.22  $\mu\text{M}$  BA were the highest among the calluses produced on cytokinin and auxin, alone and in combination (Tables 1, 2, 3 and 4). Cytokinins have been demonstrated to stimulate production of alkaloids in cultures of *Catherantus roseus* (Decendit et al. 1992); catechins, proanthocyanidins and lignins in callus cultures of *Pseudotsuga menziesii* (Zaprometov 1988). In contrast,

**Fig. 1** Callus culture and in vitro propagation of *P. amarus*.

**a** Extensive proliferation of callus on MS + 2.22  $\mu\text{M}$  BA.  
**b** Regeneration of shoot from leaf derived callus on MS + 2.27  $\mu\text{M}$  TDZ.  
**c** Proliferation of multiple shoot from the leaf derived callus on MS + 2.27  $\mu\text{M}$  TDZ.  
**d** Rooting of shoot on MS + 1.5  $\mu\text{M}$  IAA.  
**e** Regenerated plants in field conditions



**Table 5** Influence of TDZ on regeneration of shoot and accumulation of phyllanthin and hypophyllanthin in *P. amarus*

Growth regulators ( $\mu\text{M}$ ) TDZ	Callus derived from leaf explant			
	Callus respond for shoot regeneration %	Number of shoot per culture	Phyllanthin $\mu\text{g/g DW}$	Hypophyllanthin $\mu\text{g/g DW}$
0.45	55.6 $\pm$ 2.1	10.2 $\pm$ 1.6 <sup>c</sup>	235.1 $\pm$ 0.7 <sup>b</sup>	226.2 $\pm$ 0.4 <sup>b</sup>
2.27	69.2 $\pm$ 2.3	32.4 $\pm$ 1.3 <sup>a</sup>	456.4 $\pm$ 0.3 <sup>a</sup>	332.7 $\pm$ 0.2 <sup>a</sup>
4.54	51 $\pm$ 2.1	15.6 $\pm$ 1.5 <sup>b</sup>	139.5 $\pm$ 0.6 <sup>c</sup>	113.3 $\pm$ 0.8 <sup>c</sup>
9.08	42.5 $\pm$ 2.2	5.2 $\pm$ 1.0 <sup>d</sup>	128.2 $\pm$ 0.2 <sup>d</sup>	104.9 $\pm$ 0.1 <sup>d</sup>
22.71	–	C	–	–
40	–	C	–	–

The values represent the mean  $\pm$  SE calculated from three independent experiments, each based on a minimum 21 replicates. Values followed by same superscript letter were not significantly different at 5% level (DMRT)

C Only proliferation of callus

**Table 6** Phyllanthin and Hypophyllanthin content in naturally grown and in vitro raised field acclimatized plant of *P. amarus*

Plant Part	Naturally grown plant		In vitro raised field acclimatized plant	
	Phyllanthin ( $\mu\text{g/g}$ )	Hypophyllanthin ( $\mu\text{g/g}$ )	Phyllanthin ( $\mu\text{g/g}$ )	Hypophyllanthin ( $\mu\text{g/g}$ )
Leaflets	223.6 $\pm$ 0.5 <sup>a</sup>	113.2 $\pm$ 0.3 <sup>a</sup>	258.3 $\pm$ 0.3 <sup>a</sup>	128.3 $\pm$ 0.4 <sup>a</sup>
Stem	96.6 $\pm$ 0.9 <sup>c</sup>	46.9 $\pm$ 0.4 <sup>c</sup>	89.3 $\pm$ 0.5 <sup>c</sup>	52.8 $\pm$ 0.5 <sup>c</sup>
Fruit	116.6 $\pm$ 0.6 <sup>b</sup>	90.1 $\pm$ 0.6 <sup>b</sup>	106.2 $\pm$ 0.2 <sup>b</sup>	87.4 $\pm$ 0.1 <sup>b</sup>
Root	35.2 $\pm$ 0.1 <sup>d</sup>	23.2 $\pm$ 0.2 <sup>d</sup>	30.4 $\pm$ 0.6 <sup>d</sup>	19.3 $\pm$ 0.7 <sup>d</sup>

The values represent the mean  $\pm$  SE calculated from three independent experiments, each based on a minimum 21 replicates. Values followed by same superscript letter were not significantly different at 5% level (DMRT)

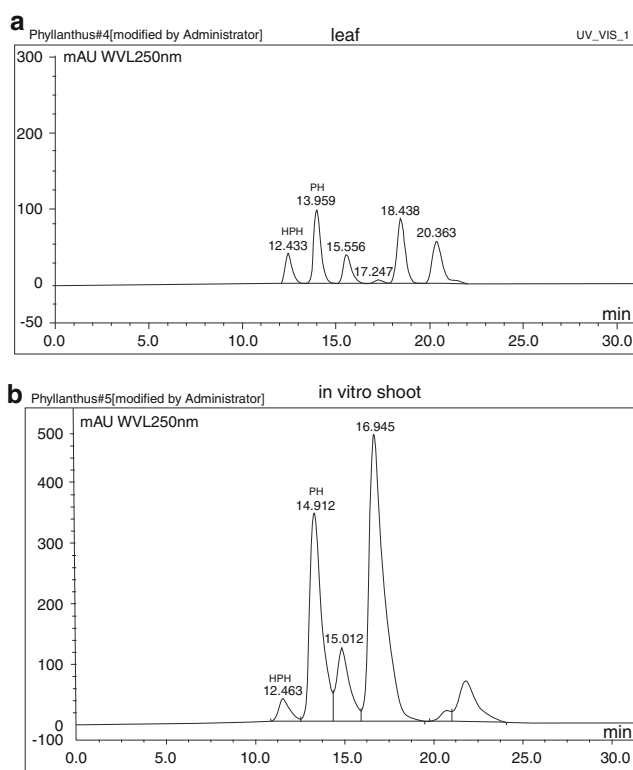
cytokinins were found to inhibit naphthaquinone formation in *Lithospermum erythrorhizon* (Fujita et al. 1981) and secoiridoid production in *Blackstonia perpurata* (Sabovljevic et al. 2006). The results of the present investigation are in agreement with the assertion that the change in the type and concentration of growth regulator may drastically reduce or increase product accumulation (Constabel 1987).

#### Regeneration of shoot and root from callus

The calluses derived from the nodal and leaflet explants and sub-cultured on the media containing IAA (0.57–28.54  $\mu\text{M}$ ) or NAA (0.5–26.85  $\mu\text{M}$ ) proliferated and produced white thin roots in the second week of culture. Maximum number of roots (11.6  $\pm$  0.3/culture) was produced from nodal explant derived callus on MS medium fortified with 5.71  $\mu\text{M}$  IAA (Table 3). Rajasubramaniam and Saradhi (1994) reported formation of roots from the surface of hypocotyl-derived callus in *P. fraternus* on B5 medium fortified with  $10^{-6}\text{M}$  BAP +  $10^{-6}\text{M}$  NAA. In the present study, the calluses developed on 2,4-D-containing media did not respond to rooting (Table 3). 2,4-D generally blocks morphogenesis and favors de-differentiation and callus development (Dicosmo and Misawa 1996). The leaf callus developed on medium with 2.26  $\mu\text{M}$  2,4-D and 2.32  $\mu\text{M}$  Kin, upon transfer to MS

medium with TDZ (0.45–9.08  $\mu\text{M}$ ) responded for shoot regeneration. The regenerative callus first turned dark green and nodular, and then these nodular structures further developed into small shootlets which attained the height of about 3–4 cm in 4 weeks after the initiation of culture (Fig. 1b, c). The medium supplemented with 0.45  $\mu\text{M}$  TDZ usually induced 8–12 shoots per culture, whereas raising the level up to 2.27  $\mu\text{M}$  TDZ resulted in an increase in the percentage of cultures responding for shoot formation (69.2%) and mean number of shoots per culture (maximum 32.4  $\pm$  1.3 shoots per culture) (Table 5; Fig. 1c). At higher levels (4.54–9.08  $\mu\text{M}$ ), however, reduction in number of shoots per culture was observed. Addition of IAA and NAA (2.5–10  $\mu\text{M}$ ) to medium containing 2.27  $\mu\text{M}$  TDZ completely inhibited shoot regeneration (data not presented). The fresh as well as sub-cultured calluses derived from leaf explants cultured on all the media except MS medium with 2.26  $\mu\text{M}$  2,4-D: 2.32  $\mu\text{M}$  Kin, when transferred onto the medium with different levels of BA, Kin and TDZ, alone or together with auxins IAA, NAA or 2,4-D, did not show shoot regeneration. None of the calluses derived from the nodal explant responded to shoot regeneration.

TDZ is a potent bioregulant of in vitro morphogenesis (Murthy et al. 1998). Studies confirm the effective use of TDZ as the sole growth regulating compound for the



**Fig. 2** HPLC chromatograph showing Phyllanthin and hypophyllanthin. **a** In natural leaf, **b** in shoot biomass obtained through 2.27  $\mu\text{M}$  TDZ treatments

induction of regeneration in many species like *Tylophora indica* (Thomas and Philip 2005), *Glycine max* (Kaneda et al. 1997) and *Fragaria* sp. (Landi and Mezzetti 2006). In the present study, only TDZ (2.27  $\mu\text{M}$ ) was effective for shoot regeneration in leaf-derived callus on MS medium with 2,4-D (2.26  $\mu\text{M}$ ) together with Kin (2.32  $\mu\text{M}$ ). These results revealed that the source of explant as well as growth regulator in the medium were critical for regenerative response in terms of induction of callus and differentiation of shoots in *P. amarus*.

#### Accumulation of PH and HPH

Biosynthesis and accumulation of metabolites in relation to organ development like root and shoot is well established and is often bound to a specific stage of plant development. Even within a single cell, a high degree of organization is observed (Wink 1987). In this study, the accumulation of PH and HPH was low in root-forming calluses of both nodal and leaf explants grown on IAA- and NAA-containing MS medium and in the roots of naturally grown plants, suggesting that roots are not a good source of PH and HPH.

The production of PH and HPH from the node-derived calluses on 2,4-D:Kin containing media was comparatively

much less and remained in the range of 10–20  $\mu\text{g/g}$  DW and 5–10  $\mu\text{g/g}$  DW, respectively (data not presented). However, only the leaf-derived calluses grown on 2,4-D- and Kin-containing media upon transfer to TDZ-containing medium showed shoot regeneration. Different quantities of PH and HPH were detected in shoot biomass obtained through TDZ treatments (Table 5). Two-fold increased production of PH ( $456.4 \pm 0.3 \mu\text{g/g}$  DW) and HPH ( $332.7 \pm 0.2 \mu\text{g/g}$  DW) was observed in the shoots obtained on 2.27  $\mu\text{M}$  TDZ-containing medium compared to that of leaflets of naturally grown plants (Tables 5 and 6; Fig. 2b). The differentiation, organ development and presence of TDZ together might be responsible for the creation of favorable cytoplasmic conditions for enhanced production of PH and HPH in the in vitro regenerated shoots. It is generally considered that plant growth regulators do not react with intermediate compounds of biosynthetic pathways but appear to change cytoplasmic conditions for product formation (Ramawat and Merrillion 1999).

TDZ has been well established as a potent cytokinin for several plants. Smith et al. (2002) reported significantly increased gland number and hypericin accumulation in shoot cultures of *Hypricum perforatum* on medium fortified with TDZ. Likewise, Liu et al. (2007) also observed increased hypericin and clustering shoots in *H. perforatum* on medium supplemented with 2.27  $\mu\text{M}$  TDZ. In contrast, in *H. sampsonii*, TDZ (0.23–0.91  $\mu\text{M}$ ) incorporation decreased hypericin levels and resulted in malformed leaves and poor shoot growth (Liu et al. 2007). The results of current study on *P. amarus* and earlier reports on *H. perforatum* and *H. sampsonii* indicate that plant growth and developmental stage are important factors in the accumulation of secondary metabolites.

#### Rooting of shoots and acclimatization

The success of in vitro regeneration depends on efficient rooting of regenerated shoots and survival of the plantlets under field conditions. The regenerated shoots of approximately 2–3 cm length failed to root on auxin free medium, whereas those cultured on MS medium fortified with either IAA or NAA (1.5–22  $\mu\text{M}$ ) rooted with variable frequency (Table 7). Catapan et al. (2002) reported spontaneous rooting in microcuttings of *P. urinaria* on medium lacking plant growth regulators. In the present study, defoliation was observed in the shoots upon transfer to root induction medium, though new leaves were produced within 4 weeks of culture. Root initiation was observed within 10–12 days in shoots cultured on IAA- or NAA (1.5–22  $\mu\text{M}$ )-containing media, but with different rooting percentage, number of roots per shoot and average length of roots. The best response was 12.7 roots per shoot, obtained on



**Table 7** Effect of IAA and NAA on in vitro rooting of shoots of *P. amarus*

Growth regulators	Shoots producing roots %	Number of root per shoot	Root length cm
IAA			
00	00	00	00
1.5	98 ± 2.3	12.7 ± 1.2 <sup>ab</sup>	5.3 ± 1.2 <sup>a</sup>
2.5	96 ± 2.5	8.3 ± 1.0 <sup>cd</sup>	5.2 ± 1.4 <sup>b</sup>
5.0	94 ± 2.4	7.5 ± 1.3 <sup>d</sup>	4.9 ± 0.9 <sup>c</sup>
10.0	89 ± 2.1	4.6 ± 1.2 <sup>e</sup>	4.7 ± 1.3 <sup>d</sup>
15.0	85 ± 2.4	3.2 ± 1.1 <sup>e</sup>	3.8 ± 1.2 <sup>e</sup>
22.0	78 ± 2.2	0.8 ± 1.2 <sup>f</sup>	4.6 ± 1.2 <sup>d</sup>
NAA			
1.5	100 ± 1.3	14.2 ± 0.9 <sup>a</sup>	2.4 ± 0.2 <sup>f</sup>
2.5	97 ± 1.9	12.4 ± 0.2 <sup>b</sup>	2.3 ± 0.3 <sup>f</sup>
5.0	94 ± 1.7	9.7 ± 0.4 <sup>c</sup>	1.9 ± 0.4 <sup>g</sup>
10.0	83 ± 1.3	7.6 ± 0.6 <sup>d</sup>	0.8 ± 0.4 <sup>h</sup>
15.0	79 ± 1.1	4.1 ± 0.8 <sup>e</sup>	0.9 ± 0.3 <sup>h</sup>
22.0	65 ± 1.6	3.2 ± 0.5 <sup>e</sup>	0.8 ± 0.4 <sup>h</sup>

The values represent the mean ± SE calculated from three independent experiments, each based on a minimum 21 replicates. Values followed by same superscript letter were not significantly different at 5% level (DMRT)

medium supplemented with 1.5 µM IAA (Fig. 1d). Shoots from this treatment had the mean longest root length of 5.3 cm (Table 7) and the highest percentage (98%) of rooting shoots. Inclusion of higher levels of IAA reduced the percentage of shoots producing roots, number of roots per shoot and root length. The shoots transferred on NAA-containing medium produced callus interspersed very thin roots. Such shoots failed to survive in soil during hardening. Callus formed at the shoot base can interfere with the connection between the shoot and root, resulting in poor root formation and on transfer, such plantlets do not survive field conditions (Quraishi et al. 1996).

The well-rooted plants were hardened for 2 weeks in the glasshouse (light 60 µmol m<sup>2</sup>s<sup>-1</sup>, humidity 80–90% and temperature 25 ± 4°C), of which almost 98% plantlets survived when transferred to field conditions (light 320 µmol m<sup>2</sup> s<sup>-1</sup>, temperature 25 ± 5°C and humidity 70–80%) (Fig. 1e). The acclimatized plants were healthy and exhibited normal growth and completed the life cycle within 4 months.

#### PH and HPH contents in in vitro explants and in vivo plant parts

Secondary metabolites are usually not distributed uniformly in different parts of the plant. Some are restricted to specific organs or tissue (Wink 1987). Among the different

parts of naturally grown plants of *P. amarus*, higher content of PH (223.6 ± 0.2 µg/g DW) and HPH (113.2 ± 0.3 µg/g DW) was recorded in leaf (Fig. 2a), followed by fruit and comparatively much less content in the stem and roots (Table 6). A similar trend was observed for content of PH and HPH in root, stem, leaf and fruit of in vitro raised field acclimatized plants and the contents were not significantly different as compared to the parts of naturally grown plants (Table 6). The results indicate that the in vitro raised plantlets on acclimatization to the natural conditions were as good as the naturally grown plants for production of PH and HPH. The shoots raised on TDZ-containing medium showed two-fold higher contents of PH and HPH. However, after rooting, these shoot-derived plantlets showed decline in PH and HPH which was almost equal to that of naturally grown plants. It is possible that IAA present in the rooting medium counteracted the effect of TDZ and inhibited the production of PH and HPH. Sabovljevic et al. (2006) reported that, though there was no qualitative difference between the plants of *Blackstonia perfoliata* growing in the wild and those cultured in vitro, the levels of secondary metabolites secoiridoids, gentiopin, swertiamarin and sweroside differed in them.

To our knowledge, this is the first report on comprehensive analysis of the effects of auxins and cytokinins on the production of phyllanthin and hypophyllanthin in callus cultures, shoots and roots in comparison to in vitro raised—field acclimatized plants and naturally grown plants of *P. amarus*. As indicated by the variables tested in this study, the shoots regenerated from leaf-derived callus on TDZ containing MS medium can be one of the best sources of hepatoprotective phyllanthin and hypophyllanthin. This protocol may be useful for their scaling up using bioreactor-based production.

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