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RESEARCH NOTE

Biosynthesis and accumulation of a medicinal compound, Picroside-I, in cultures of *Picrorhiza kurroa* Royle ex Benth

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Abstract Establishment of callus cultures and plant regeneration from different explants coupled with estimation of Picrosides in morphogenetically different developmental stages showed that Picroside-I accumulates in shoot cultures of *Picrorhiza kurroa* with no detection of Picroside-II. The Picroside-I content was 1.9, 1.5, and 0.04 mg/g in leaf discs, stem and root segments, respectively. The Picroside-I content declined to almost non- detectable levels in callus cultures derived from leaf discs, stem segments with no change in Picroside-I content in root segments or calli derived thereof. The biosynthesis and accumulation of Picroside-I started in callus cultures differentiating into shoot primordia and reached to the concentrations comparable to original explants of leaf discs and stem segments in fully developed shoots with contents of 2.0 and 1.5 mg/g, respectively. The shoots formed from root-derived callus cultures were relatively slow in growth as well as the amount of Picroside-I content was comparatively low (1.0 mg/g) compared to shoots derived from callus cultures of leaf and stem segments, respectively. The current study concludes that the biosynthesis and accumulation of Picroside-I is developmentally regulated in different morphogenetic stages of *P. kurroa* tissue cultures.

Keywords Shoots · Callus · Regeneration · Morphogenesis · Picroside · Indirect organogenesis

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Abbreviations

IBA	Indole-3-butyric acid
2,4-D	2,4 Dichlorophenoxyacetic acid
KN	Kinetin
BA	6-Benzyladenine

Picrorhiza kurroa Royle ex Benth (Family: Scrophulariaceae) is a medicinal herb, mainly found in the North-Western Himalayan regions of India at altitudes of 3,000–4,300 m. *P. kurroa* is a well-known herb in the Ayurvedic system of medicine and has traditionally been used to treat disorders of the liver and upper respiratory tract, reduce fevers, and to treat dyspepsia, chronic diarrhea, and scorpion sting. The active constituents are obtained from the shoots, roots and rhizomes of plant. *P. kurroa* is a high value medicinal herb due to rich source of hepatoprotective metabolites, Picroside-I and Picroside-II and other metabolites like Picroside-III, Picroside-IV, Apocynin, Androsin, Catechol, Kutkoside, etc. (Weinges et al. 1972; Stuppner and Wagner 1989). The medicinal importance of *P. kurroa* is due to its pharmacological properties like hepatoprotective (Chander et al. 1992), antioxidant (particularly in liver) (Ansari et al. 1988), antiallergic and antiasthamatic (Dorch et al. 1991), anticancerous activity particularly in liver (Joy et al. 2000) and immunomodulatory (Gupta et al. 2006). A hepatoprotective drug formulation, Picroliv has been prepared from the extracts of *P. kurroa* (Ansari et al. 1991; Dwivedi et al. 1997). Picroside-I is the major ingredient of Picroliv and, therefore, makes this compound a highly valued secondary metabolite of *P. kurroa*. The *P. kurroa* plants have been recklessly collected from its natural habitat, thereby, reducing its populations and putting it under the

category of endangered plant species. The only way to overcome pressure from natural habitat of *P. kurroa* is to optimize cell cultures conditions for large-scale production of its metabolites in vitro and to conserve quality germplasm. However, the production of metabolites through tissue cultures of *P. kurroa* requires thorough understanding of their biosynthesis and accumulation in different morphogenetic tissue culture stages so as to identify a particular stage which is most suitable and amenable for in vitro culture coupled with biosynthesis and accumulation of Picrosides.

The biosynthesis of Picroside-I and Picroside-II is reported to occur differentially in shoots and roots of *P. kurroa* wherein the Picroside-I accumulates preferentially in shoots and Picroside-II in the roots of field grown plants of *P. kurroa* (Chauhan and Sood unpublished). The differential accumulation of Picroside-I and Picroside-II in shoots and roots of field grown plants indicates that the biosynthesis of both these metabolites occurs in specialized cell types. However, what determines the biosynthesis of Picroside-I in the shoots and that of Picroside-II in the roots is not known. Moreover, the biosynthesis and accumulation of Picroside-I and Picroside-II occur in *P. kurroa* at high altitudes and that too during a particular time of a season, which complicates the process of understanding biology of their biosynthesis. The cell cultures offer a suitable biological system with a controlled environment wherein the morphogenetic events can be regulated by manipulating the levels of growth hormones in the nutrient medium resulting in rapid production of plant metabolites of pharmaceutical importance (Ray and Jha 2001; Tanaka et al. 1995). Tissue cultures of *P. kurroa* have been done for its rapid multiplication (Lal et al. 1988; Upadhyay et al. 1989). However, there is no information pertaining to biosynthesis and accumulation of Picroside-I and Picroside-II in tissue cultures of *P. kurroa.* We report herein the identification of developmental events in the tissue cultures of *P. kurroa* starting from original explant such as leaf discs, stem and root segments, passing through different morphogenetic stages such as de-differentiation of original explants into a callus mass and then re-differentiation of callus into shoot primordia and fully developed shoots and roots. We also report on dynamics of Picroside-I and Picroside-II biosynthesis and accumulation in morphogenetically different tissue culture stages of *P. kurroa.*

For initiating tissue cultures, the *P. kurroa* plants were procured from the Himalayan Forest Research Institute, Panthaghati, Shimla, H.P., India and planted in pots in a polyhouse at the experimental area of the Department of Biotechnology and Bioinformatics, Jaypee University of Information Technology, Waknaghat, India. Shoot apices were surface sterilized in 0.5% Bavistin and 0.1% Mercuric Chloride followed by 4–5 washings in sterile water and cultured on Murashige and Skoog (MS) medium (Murashige and Skoog 1962) supplemented with indole-3-butyric acid (IBA) (2 mg/l) and kinetin (KN) (3 mg/l), which was found suitable for in vitro shoot multiplication of *P. kurroa* (Sood and Chauhan 2009).

Leaf discs (1 cm diameter), stem segments (only stem ~ 1–1.5 cm) and root segments (~1–1.5 cm) were excised from 4 to 5 weeks old in vitro growing plantlets of *P. kurroa* and cultured on calli induction medium for their de-differentiation into callus cultures (Fig. 1a). The medium consisted of MS + 2,4 dichlorophenoxyacetic acid (2,4-D) (2 mg/l), IBA (0.5 mg/l), sucrose 3% (w/v) and solidified with agar (0.8%) for their de-differentiation into callus cultures (Fig. 1a). The medium was poured into 150 ml Erlenmeyer flasks (Borosil, India) by dispensing 40 ml media in each flask and inoculated with 7–10 explants/flask. The cultures were incubated at 16 h photoperiod provided by cool white fluorescent light (3,000 lux) at 15 ± 1°C in a plant tissue culture chamber.

Three to four weeks after inoculation the explants were completely transformed into callus mass with a cream colour. These calli were then transferred onto shoot regeneration medium consisting on the same basal composition of the calli induction medium but supplemented with 6-benzyladenine (BA) (2 mg/l), KN (1.0 mg/l) and IBA (1.0 mg/l). The cultures were incubated at 15 ± 1°C and 16 h photoperiod (3,000 lux).

The estimation of Picroside-I and Picroside-II was carried out by High Performance Liquid Chromatography (HPLC) analysis for which three tissue samples were collected from each morphogenetic stage of tissue cultures of *P. kurroa*. The quantification of Picroside-I and Picroside-II was carried by reverse phase (HPLC Waters 515) through C18 (5 μm) 4.6 × 250 mm Waters Symmetry Column using PDA detector (Waters 2996). Fresh samples of different tissue culture stages were ground separately in liquid nitrogen and suspended in 80% methanol. The sample mixture was vortexed and kept overnight at room temperature. Following day, the samples were centrifuged at 10,000 rpm for 10–15 min and the supernatant was filtered through 0.22 μm filter. The filtrate was diluted 10× and 20× and injected into above mentioned column. Two solvent systems were used for running the test samples i.e. Solvent A (0.05% trifluoroacetic acid) and Solvent B (1:1 methanol/acetonitrile mixture). Solvent A and B were used in the ratio of 70:30 (v/v). The column was eluted in the isocratic mode with a flow rate of 1.0 ml/min. The Picroside-I and Picroside-II were detected at absorbance of 270 nm wavelength. The cycle time of analysis was 30 min at 30°C. The compounds were identified on the basis of their retention time and comparison of UV spectra with the authentic standards procured from ChromaDex, Inc.

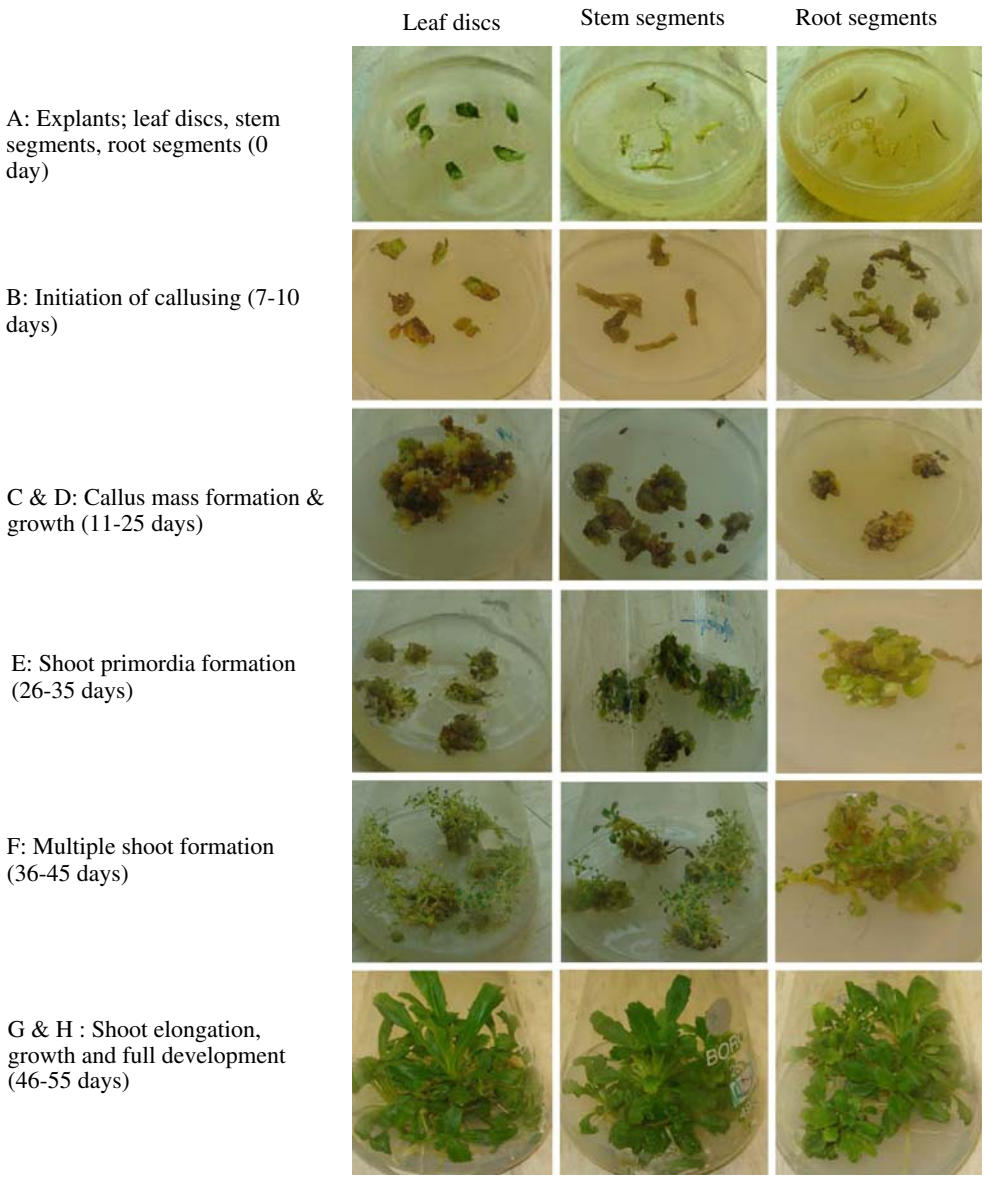

Fig. 1 Morphogenetic developments in tissue cultures of *P. kurroa* from three explants; leaf discs, stem segments and root segments

The estimation of Picroside-I and Picroside-II in different explants before culturing revealed that the leaf and stem segments contained higher amounts of Picroside-1 with 1.9 and 1.5 mg/g fresh wt., respectively, whereas root segments contained negligible amount of Picroside-I (0.04 mg/g fresh wt.). The amount of Picroside-II was very low (0.002 mg/g fresh wt.) in all three explants. The three explants started de-differentiating into a callus mass after 6–7 days of incubation and concomitantly the amount of Picroside-I also started declining in callusing explants of leaf and stem segments (Fig. 2). The amount of Picroside-I was low in the root explants and remained low in the root-derived callus cultures. The explants were transformed completely into a callus mass after 4 weeks of incubation and correspondingly the Picroside-I content was also reduced to almost non-detectable level in callus cultures with 0.026, 0.020 and 0.01 mg/g from leaf, stem segments and root explants, respectively (Fig. 2). After the callus formation from all the three explants, the callus cultures were transferred to a regeneration medium for differentiating into shoots. The callus cultures started differentiating into shoot primordia after 5 weeks and fully developed shoots were formed after 8 weeks of the initial inoculation in calli induction medium culturing. The callus cultures showing the formation of shoot primordia and those with fully developed shoots were assessed for Picroside-I biosynthesis and accumulation (Fig. 1e–h). The shoot primordia forming callus cultures started showing the

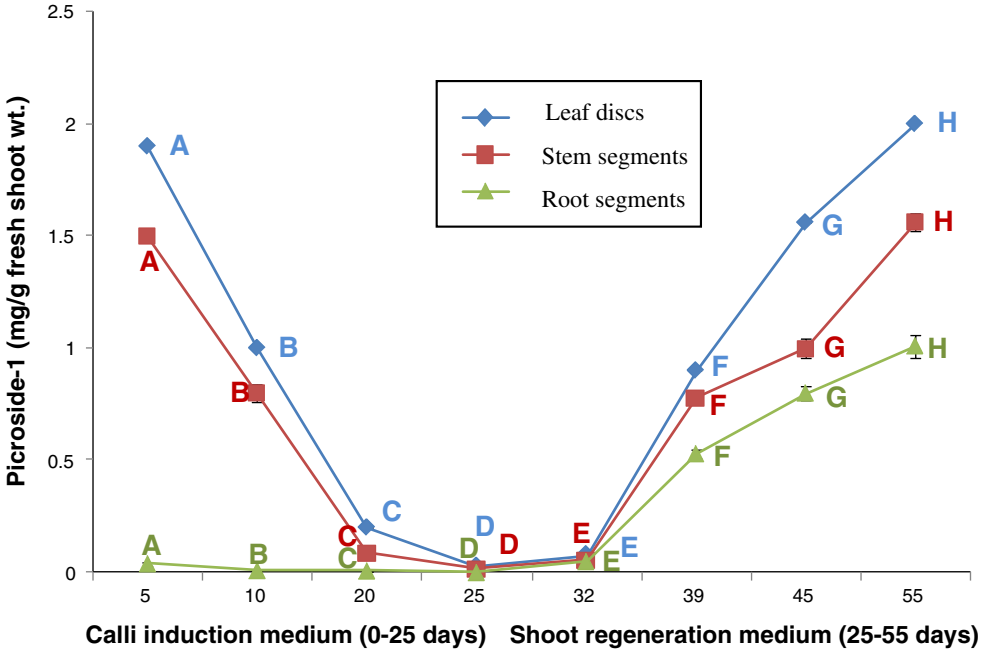

Fig. 2 Biosynthesis and accumulation of Picroside-I in different morphogenetic tissue culture stages (a–h as in Fig. 1) of *P. kurroa*

biosynthesis and accumulation of Picroside-I (Fig. 2). In contrast the amount of Picroside-I was negligible in root segments as well as in the callus cultures derived from root explants, thereby, implying that the biosynthesis of Picroside-I does not occur in root cells of in vitro formed roots. However, the amount of Picroside-I increased gradually after transferring the calli developed from the three explants to the regeneration medium till the complete development of shoots. Interestingly, the callus cultures derived from root segments and differentiating into shoot primordia also started showing the biosynthesis and accumulation of Picroside-I. Two weeks after inoculation, the amount of Picroside-I was slightly higher though at non-significant levels in shoot primordia developed by indirect organogenesis from leaf segments (0.08 mg/g fresh wt.) followed by shoots developed from stem and root segments (0.06 and 0.05 mg/g fresh wt., respectively) After 4 weeks of in vitro inoculation, the fully developed shoots from callus cultures of leaf segments contained the highest amount of Picroside-I ($\sim$2.0 mg/g fresh wt.) followed by 1.5 and 1.0 mg/g fresh wt. in shoots regenerated from callus cultures derived from stem and root segments, respectively. The differential accumulation of Picroside-I content in shoots derived from callus cultures of leaf, stem and root segments may be due to the differences in growth and development of shoots derived from these explants. The shoots regenerated from root derived callus cultures were comparatively slow in growth and less developed. These observations indicate that the stage of growth and development in cell cultures is an important factor in the regulation of biosynthetic pathways. The biosynthesis and accumulation of secondary metabolites have been found to vary among plant parts and morphological stages as well as different morphogenetic tissue culture stages. The ellagic acids present in *Rubus chamaemorus* plants was 3 times lower in shoot cultures and over 10 times in callus cultures (Thiem and Krawczyk 2003). Similarly in *Salvia officinalis* (Grzegorczyk et al. 2007) and *Roseb rosemary* (Caruso et al. 2000) in vitro cultures, the abietane diterpene antioxidants (carmisol and carnosic acid) were present only in shoot cultures and not in callus, suspension or hairy roots. Similar observations have been made in the biosynthesis of vindoline in *Catharanthus roseus*, wherein the metabolite does not accumulate in the callus cultures but occurs specifically in the shoots (Aerts and De Luca 1992). The artimisinin production has been minimal in undifferentiated callus and cell suspension cultures of *Artemisia annua* (Liu et al. 2003). A certain degree of differentiation of shoot cultures was a prerequisite for artimisinin biosynthesis (Paniego and Giulietti 1996). The lack of biosynthesis of Picroside-I in undifferentiated callus cultures of *P. kurroa* can be attributed to the absence of proper cell organization and programming of cell machinery involved in the biosynthesis of Picroside-I. The importance of chloroplasts in the biosynthesis of Picroside-I in leaf and stem segments and fully developed shoots cannot be ruled out because the callus cultures and in vitro formed root segments lack chloroplasts as well as Picroside-I content. The modification of relative biosynthesis to degradation ratios of a desired product can also influence the final levels of a desired compound in culture. However, whether biosynthesis of Picroside-I is shut down in callus cells or there is degradation of Picroside-I in callus cells remain to be understood. The observation that biosynthesis and

accumulation of Picroside-I occurs preferentially in differentiated shoots of *P. kurroa* and not in de-differentiated callus cultures, whereas there is no biosynthesis of Picroside-II neither in differentiated shoot/root cultures nor in de-differentiated callus cultures of *P. kurroa* opens up several avenues to investigate biology of Picrosides biosynthesis in more detail. The Picroside-I and Picroside-II are iridoid glycosides belonging to monoterpene class of terpenoids (Negi et al. 2008) and organogenesis has often been used as a means of inducing monoterpene production in tissue cultures, which otherwise fail to produce monoterpenes as long as they are maintained in morphologically undifferentiated state, e.g. valepotriate production was increased by the induction of root morphogenesis in callus cultures of *Valeriana officinalis* and positive correlation was observed between the levels of differentiation and valepotriate production (Shrivastava et al. 2006). The results of current study indicate that the in vitro cultured shoots of *P. kurroa* possess biosynthetic potential for Picroside-I production, which can be exploited for production of Picroside-I on a large scale under proper growth conditions.

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