3 In Vitro Approaches for Conservation and Sustainable Utilization of *Podophyllum hexandrum* and *Picrorhiza kurroa*: Endangered Medicinal Herbs of Western Himalaya

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Abstract

The western Himalaya is a rich repository of unique plants that are valued for their medicinal properties. Many of these plants are extensively utilized in pharmaceutical industries, and there is a huge global demand for them. Since most of these plants have become either rare, threatened, or endangered, there is an urgent need to conserve them. *Podophyllum hexandrum* and *Picrorhiza kurroa* are two endangered medicinal herbs that are being ruthlessly uprooted for their active principles, i.e., podophyllotoxins and picrosides, respectively. Hence, different plant tissue culture approaches have been employed for their conservation and sustainable utilization. The successes achieved till date in these approaches have been reviewed in the present article.

Keywords

Cell suspension • Genetic transformation • Micropropagation • *Podophyllum hexandrum* • *Picrorhiza kurroa* • Secondary metabolite production

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3.1 Introduction

The entire stretch of mountainous terrains extending from southwest Afghanistan, northern Pakistan, northwestern India, and western parts of Nepal constitutes the "Western Himalayas." In India, the region spans through the states of Jammu and Kashmir, Himachal Pradesh, and Uttarakhand. The region is characterized by remarkable altitudinal gradients and distinct climatic zones that range from tropical at the base of the mountains to permanently snow clad alpine regions beyond the tree line. The life forms that inhabit each of these altitudinal gradients differ markedly and contribute toward the amazing natural wealth of the western Himalayan biodiversity. The region being comparatively drier than the eastern parts of Himalayas supports various flora and fauna that are distinctively different from their eastern counterparts. These serve as rich repositories of medicinal bioresources. Many of these medicinally important plants are specifically confined to unique eco-geographical niches and suffer from the risks of habitat degradation and climate change. As a result, several valuable plant species have been depleted from nature. With increasing popularity of herbal medicines in developed countries (British Medical Association 1993; Najar and Agnihotri 2012), the plants are also suffering from tremendous overexploitation. Thus, many plants of western Himalaya are becoming rare, threatened, endangered or critically endangered and figure prominently in the IUCN Red List (Verma et al. 2012). *Podophyllum hexandrum* Royle (Indian mayapple or Bankakdi) of family Berberidaceae (Fujii 1991; Giri and Narasu 2000; Kushwaha et al. 2007) and *Picrorhiza kurroa* Royle ex Benth. (Kutki) of family Scrophulariaceae are two such endangered medicinal herbs of western Himalaya (Nayar and Sastry 1990; Kushwaha et al. 2007; CITES 2007).

Of the two plants, *P. hexandrum* is acclaimed for podophyllotoxins or aryltetralin lignans present in the roots and rhizomes of the plant (Jackson and Dewick 1984; Liu et al. 2015). The compounds are known for their anticancer, antifungal, and immunomodulatory properties (Kamil and Dewick 1986; Broomhead and Dewick 1990; Canel et al. 2000; Lerndal and Svensson 2000; Ganie et al. 2010; Kushwaha et al. 2010). Podophyllotoxins and their derivatives are extensively used as precursors for the synthesis of commercial anticancer drugs such as VP-16-213 (etoposide Vumon®), VM-26 (teniposide) and etopophos®, Pod-Ben-25, Condofil, Verrusol, Warticon, GL331, Top 53, NK 611, and CPH 82 (Uden et al. 1989; Huang et al. 1996; Pagani et al. 1996; Gordaliza et al. 2004; Liu et al. 2004; Kharkwal et al. 2008; Nagar et al. 2011; Bhattacharyya et al. 2012; Kumar et al. 2015). These are approved drugs for treating lung and testicular cancers, leukemia, psoriasis, and rheumatoid arthritis (Staheliin and Von Warburg 1991; Liu et al. 2004; Chen et al. 2007). Podophyllotoxins are also used for the treatment of venereal warts (Yanofsky et al. 2012). These usages have led to ruthless uprooting and overharvesting of the underground parts of the plant to the extent that it has become endangered (Gupta and Sethi 1983; Nayar and Sastry 1990; Airi et al. 1997). Hence, the plant now figures prominently in the IUCN Red List and demands immediate and urgent conservation.

On the other hand, *P. kurroa* is known for its hepatoprotective (Chander et al. 1990; Sinha et al. 2011), anticancer, antidiabetic, cardioprotective, anticholestatic, antiulcerogenic, antiasthmatic, anti-inflammatory, and antidepressant properties (Singh et al. 1993; Joy et al. 2000; Ram 2001; Chauhan et al. 2008; Husain et al. 2009; Patani et al. 2012; Navade et al. 2013). These properties are primarily due to "kutkin," the main active constituent that accumulates predominantly in the roots and rhizomes of the plant. Kutkin is a mixture of two major C-9-iridoid glycosides, picroside I (6-O-trans cinnamoyl catalpol) and picroside II (6-vanilloyl catalpol) (Jia et al. 1999; Mondal et al. 2013). The detailed chemical structures of both picroside I and picroside II were elucidated by Kitagawa et al. (1971) and Weinges et al. (1972). *P. kurroa* is highly sought by Indian herbal industries wherein 5000 tonnes of the plant constitute the annual demand. This is in sharp contrast to the annual supply of *P. kurroa*, which is less than 100 tonnes (Kumar 2006). Thus, the plant is suffering from ruthless and illegal overharvesting from its natural habitats. Natural populations of the plant is also rapidly dwindling due to habitat specificity, restricted distribution, overgrazing, landslides, poor natural regeneration, and anthropogenic activities (Chandra et al. 2006; Bantawa et al. 2009). Presently, the plant is categorized as "endangered" in the Appendix II of the "Convention on International Trade in Endangered Species of Wild Fauna and Flora" (CITES 2000) and the "International Union for Conservation of Nature and Natural Resources" (Leaman 2007). Hence, there is an urgent need to conserve the plant.

While there are various methods of plant conservation, tissue culture is popularly used in case of valuable but rare, endangered, and threatened (RET) plants. The method has been extensively used as an alternative route for rapid clonal multiplication of valuable germplasm and their reintroduction in nature (Basavaraju 2005; Narula et al. 2007; Srivastava et al. 2010; Pant 2013). Tissue culture has also been used to produce secondary metabolites in root, callus, and cell suspension cultures. However, these approaches require extensive optimization, and the success rates are often limited. In this regard, the efforts made towards the conservation and sustainable utilization of *Podophyllum hexandrum* and *Picrorhiza kurroa* are reviewed in the present article.

3.2 *Podophyllum hexandrum*

The major focus of *P. hexandrum* tissue culture has been the development of in vitro systems for podophyllotoxins production. However, mass propagation of the plant through shoot multiplication, somatic embryogenesis, and caulogenesis has also been attempted by a few workers (Table 3.1).

3.2.1 Micropropagation

Till date, only seedlings generated from zygotic embryos were used for shoot multiplication (Nadeem et al. 2000). Germination of excised embryos on Murashige

Table 3.1 Efforts made for in vitro regeneration and podophyllotoxin production in *Podophyllum hexandrum*

Sr. no.	Explant	Medium	Response	References
1	Roots	B5 + 2% coconut water, 4% sucrose, and 4 mg l^{-1} NAA	Callus and cell suspension	Uden et al. (1989)
2	Rhizome (callus derived from rhizome)	B5 + 2% coconut water, 4% sucrose, 4 mg l^{-1} NAA and 2.5 mM each of phenylalanine, coniferin, tyrosine, cinnamic acid, caffeic acid, coumaric acid, and ferulic acid independently	Podophyllotoxins increased by 12.8-fold in cell suspension developed on coniferin supplemented medium	Uden et al. (1990)
3	Roots	B5 + 4% sucrose, 4 mg l^{-1} NAA and 3 mM of β-cyclodextrin-complexed coniferyl alcohol or noncomplexed coniferyl alcohol/coniferin	Higher accumulation of podophyllotoxins in cell suspension fed with 3 mM of β-cyclodextrin-complexed coniferyl alcohol and water-soluble coniferin	Woerdenbag et al. (1990)
4	Roots	B5 + 0.1–1.2 mg l^{-1} 2,4-D, 0.2–2.0 mg l^{-1} GA_3 and 0.05–1.0 mg l^{-1} BA	Callus culture	Heyenga et al. (1990)
			Podophyllotoxin, 4′-demethylpodophyllotoxin and podophyllotoxin-4-O-glucoside at levels similar to the original explants	
5	Zygotic embryos	(i) MS + 2.0 μM BA and 0.5 μM IAA	(i) Callusing and somatic embryogenesis	Arumugam and Bhojwani (1990)
		(ii) MS medium + 6% sucrose or 2.5 μM NAA	(ii) Further development and maturation	
		(iii) MS basal	(iii) Somatic embryo germination	
6	Zygotic embryos	(i) MS + either 0.5 μM or 10 μM 2,4-D and 1 μM BA	(i) Callus maintenance	Arumugam and Bhojwani (1994)
		(ii) MS + 2.5 μM NAA	(ii) Cotyledonary embryo development	
		(iii) MS basal	(iii) Embryo germination	

7	Cell suspension	Culture medium + cyclodextrin/cyclodextrin-complexed deoxypodophyllotoxin/deoxypodophyllotoxin	Maximum podophyllotoxin and β-D-glucoside of podophyllotoxin accumulation (2.87 % on a dry weight basis) in deoxypodophyllotoxin-fed cultures after 9 days	Uden et al. (1995)
8	Zygotic embryos	(i) MS basal	(i) Germination within 1 week	Nadeem et al. (2000)
		(ii) MS medium + 1 μM IAA and 1 μM BA	(ii) Multiple shoots in 4–5 weeks	
		(iii) MS medium + 2–4 μM IAA and 1 μM BA	(iii) Rooting of micro-shoots	
		(iv) MS 5mM NAA and 0.5 mM BA	(iv) Somatic embryos from callus after 16 weeks of culture	
9	Roots	(i) ½ MS + 1 mg l^{-1} 2,4-D, 0.1 mg l^{-1} BAP, and 1 mg l^{-1} GA_3	(i) Callusing	Giri et al. (2001)
		(ii) MS + 1 mg l^{-1} 2,4-D and 0.1 mg l^{-1} BAP	(ii) Suspension culture	
		Agrobacterium rhizogenes strains A4, K599, and 15834		
10	Roots	(i) MS + 2 mg l^{-1} IAA and 5 g l^{-1} activated charcoal	(i) Friable callus culture	Chattopadhyay et al. (2001)
		(ii) MS + 2 mg l^{-1} IAA, 10 g l^{-1} PVP and 1.5 mg l^{-1} pectinase	(ii) Suspension culture	
11	Roots	(i) MS + 2 mg l^{-1} IAA and 5 g l^{-1} activated charcoal	(i) Friable callus culture	Chattopadhyay et al. (2002a)
		(ii) MS + 2 mg l^{-1} IAA, 10 g l^{-1} PVP, and 0.005 mg l^{-1} pectinase	(ii) Suspension culture	
12	Roots	(i) MS + 11.4 μM IAA, 10 g l^{-1} PVP, 1.5 mg l^{-1} pectinase, and 30 g l^{-1} glucose	(i) Suspension culture initiation	Chattopadhyay et al. (2002b)
		(ii) MS + 5 g l^{-1} PVP and 30 g l^{-1} glucose	(ii) Suspension culture maintenance	
13	Roots	(i) MS +2 mg l^{-1} IAA and 5 g l^{-1} activated charcoal	(i) Callus culture	Chattopadhyay et al. (2003a)
		(ii) MS + 2 mg l^{-1} IAA, 10 g l^{-1} PVP, and 1.5 mg l^{-1} pectinase	(ii) Suspension culture	

(continued)

Table 3.1 (continued)

Sr. no.	Explant	Medium	Response	References
14	Roots	Medium same as Chattopadhyay et al. (2001)	Podophyllotoxin accumulation in batch mode was 21.4 g l^{-1} and 13.8 mg l^{-1} after 24 and 26 days	Chattopadhyay et al. (2003b)
		Culturing in a 3 L stirred-tank bioreactor under low-shear condition in batch and fed batch modes of operation		
15	Cell cultures	Cytotoxicity of podophyllotoxins produced in cell culture tested using human breast cancer cell line (MCF-7)	50 % inhibition by 1 nM podophyllotoxin, provided it was applied during the beginning of cell growth	Chattopadhyay et al. (2003c)
16	Cell cultures	Bioreacter scale culture in cell retention cultivation mode	Podophyllotoxin accumulation in batch mode was 21.4 g l^{-1} and 13.8 mg l^{-1} after 24 and 26 days, respectively	Chattopadhyay et al. (2004)
17	Excised embryo culture	B5 + GA_3	Germination and en masse plant production	Kharkwal et al. (2004)
18	Zygotic embryos	LS (in dual shake flasks and dual bioreactors)	Suspension culture	Lin et al. (2003a, b)
19	Zygotic embryos	B5 and MS	Root cultures	Sagar and Zafar (2005)
20	Root segments	(i) Half strength B5 + 0.5–1.5 mg l^{-1} 2, 4-D, and 0.2–1.0 mg l^{-1} BA	(i) Callus initiation	Sultan et al. (2006)
		(ii) MS + 0.5 mg l^{-1} BA and 1 mg l^{-1} IAA	(ii) Shoot proliferation	
		(iii) MS + 0.5–1.0 % activated charcoal and 0.5–2.0 mg l^{-1} NAA	(iii) High rooting efficiency	
		(iv) Jiffy pots containing sand, soil, and vermiculite in 1:1:1 ratio	(iv) Hardening under polyhouse conditions	
21	Zygotic embryos	(i) Solid nutrient agar slab	(i) Embryo germination	Ahmad et al. (2007)
		(ii) MS + 0.5–2 μM NAA, 1–2.5 μM BAP, and 2.5 μM GA_3	(ii) Callusing	

22	Excised embryos	MS + 1 mg l^{-1} IAA, 0.5 mg l^{-1} BA, and 1 % activated charcoal	Maximum plantlet growth	Sultan et al. (2009)
23	Juvenile and mature explants from in vitro-grown seedlings	B5 and MS + different concentrations of 2,4-D, GA_3, and 6-BA	Callus and cell suspension	Majumder and Jha (2007; 2009)
24	Zygotic embryo and rhizomes	MS + different concentrations of IBA, GA_3, hydroquinone, and activated charcoal	Root culture	Li et al. (2009)
25	Rhizome explants	(i) MS + 11.42 μM IAA	(i) Multiple shoot formation	Chakraborty et al. (2010)
		(ii) MS + 2.68 μM NAA and 11.1 μM BAP	(ii) Multiple shoot formation	
		(iii) Half strength liquid MS + 100 μM IBA	(iii) Root formation	
26	Shoots derived from zygotic embryo	WPM + 1.5 mg l^{-1} IAA and 0.5 mg l^{-1} NAA	In vitro rooting	Guo et al. (2012)
27	Callus developed by Majumder and Jha (2009)	Half strength liquid B5 + 1 % (w/v) sucrose, 0.1 mg l^{-1} BAP, 1 mg l^{-1} each of 2,4-D, and GA_3	Cell suspension	Majumder (2012)
28	Mature leaves	(i) MS + 2.68 μM NAA and 8.88 μM BAP	(i) Callus culture	Bhattacharyya et al. (2012)
		(ii) MS + 60 mM total nitrogen, 1.25 mM potassium dihydrogen phosphate, 6 % glucose, and 11.41 μM IAA	(i) Suspension culture	
29	Excised zygotic embryos	MS + 1.5 mg l^{-1} 2,4-D	Callus establishment and transformation	Rajesh et al. (2013)
		A. tumefaciens strains LBA4404, EHA101, and EHA105		
30	Zygotic embryos	(i) Three fourth strength MS + 3 g l^{-1} PVP and 4 % sucrose	(i) Callusing and somatic embryogenesis	Rajesh et al. (2014a)
		(ii) Three fourth strength MS + 1 mg/L ABA, 3 g l^{-1} PVP, and 4 % sucrose	(ii) Maturation of somatic embryos	

(continued)

Table 3.1 (continued)

Sr. no.	Explant	Medium	Response	References
31	Zygotic embryos	(i) MS + 0.6 mg l^{-1} GA_3	(i) Germination of zygotic embryos	Rajesh et al. (2014b)
		(ii) MS + 5 mg l^{-1} GA_3	(ii) Direct somatic embryogenesis	
		(iii) MS + 1.5 mg l^{-1} 2,4-D	(iii) Callusing	
		(iv) MS + 1.5 mg l^{-1} each of 2,4-D, NAA, 0.5–2.5 mg l^{-1} picloram, or 0.5–2.5 mg l^{-1} ABA	(iv) Indirect somatic embryogenesis	
		(v) MS + 5 mg l^{-1} GA_3	(v) Germination of somatic embryos	
32	Shoot, leaf and root segments	(i) Half strength MS + 3 mg l^{-1} IBA and 2% sucrose	(i) Adventitious root development by root explant only	Rajesh et al. (2014c)
		(ii) Half strength MS basal + 6% sucrose	(ii) Maximum biomass and podophyllotoxin accumulation	

and Skoog (MS) medium, supplemented with 1 μM each of IAA (Indole-3- acetic acid) and BA (6-Benzyl adenine), and the use of seedlings for multiple shoot formation was reported by Nadeem et al. (2000). Although different concentrations of IAA were combined with 1 μM BA for rooting of the micro-shoots, only MS medium supplemented with 0.5 uM IAA evoked the best response. The rooted plantlets were finally transferred to pots containing vermiculite and hardened successfully. Another group reported the employment of "excised embryo culture" for extraction and germination of zygotic embryos on B5 medium (Gamborg et al. 1968) supplemented with GA_3 (gibberellic acid). The workers achieved 89.14 % germination from both mature as well as immature zygotic embryos but these were not utilized for in vitro shoot multiplication. Rather, seedling-raised plants were generated en masse and transferred to soil under green house conditions (Kharkwal et al. 2004).

On the other hand, somatic embryogenesis has been the focus of different groups working on *P. hexandrum* tissue culture. In this regard, Arumugam and Bhojwani (1990) were the first to report indirect somatic embryogenesis from excised zygotic embryos. The workers achieved both callusing followed by somatic embryogenesis on half strength MS medium supplemented with 2 μM BA and 0.5 μM IAA. The differentiated globular somatic embryos were multiplied on the same medium but these failed to mature further. Therefore, 6 % sucrose or 2.5 μM NAA (naphthalene acetic acid) was used in MS medium to support the normal development and maturation of zygotic embryos. However, basal MS medium was required for somatic embryo germination. Optimal somatic embryogenesis at 25 °C but their suppression under light and higher temperatures was also reported. In a separate study, the researchers maintained the embryogenic calli for 3 years on basal MS medium containing 10 μM 2,4-D (2,4-dichlorophenoxy acetic acid) and 1 μM BA or 0.5 μM 2,4-D (Arumugam and Bhojwani 1994). However, the calli had to be subcultured on MS medium containing 2.5 μM NAA for further development into somatic embryos and their germination on basal MS medium. Another group used radicals and cotyledonary leaves of germinated somatic embryos for callus initiation on basal MS medium supplemented with different concentrations of NAA, BA, and GA_3. Somatic embryogenesis was initiated only when the calli were transferred to MS medium containing 5.0 μM each of NAA and BA (Nadeem et al. 2000). After a long gap of 14 years, Rajesh et al. (2014a) achieved both direct and indirect somatic embryogenesis, wherein the indirect or callus-mediated pathway led to induction of high frequency of somatic embryos on basal MS medium. However, supplementation of 5.0 mg l^{-1} GA_3 was required for 79 % normal somatic embryo germination. When direct somatic embryos were further cultured on MS medium supplemented with 1.5 mg l^{-1} 2, 4-D in dark, callus development occurred and 1.8 mg g^{-1} dry weight podophyllotoxins were produced per 1.2 g of callus. The amount recorded was higher than that of field grown plants. In a parallel study, Rajesh et al. (2014b) developed an efficient method of indirect somatic embryogenesis and plantlet regeneration from zygotic embryos cultured on three fourth strength MS medium supplemented with 3.0 g l^{-1} polyvinylpyrrolidone (PVP) and 4 % sucrose in dark. The somatic embryos matured in the presence of 1.0 mg l^{-1} ABA (abscisic acid), 3.0

g l^{-1} PVP, and 4 % sucrose but highest germination (91.1 %) occurred when 1.0 mg l^{-1} GA_3 was present. Accumulation of podophyllotoxins (2.8 mg l^{-1}) in the somatic embryos was highest when sucrose was increased to 8 %. The plantlets were also hardened in growth chamber.

Besides somatic embryogenesis, caulogenesis was attempted by a few workers. In this regard, Sultan et al. (2006) employed root segments of in vitro-grown seedlings for initiation and proliferation of callus on B5 medium. Supplementation of MS medium with 0.5–5.0 mg l^{-1} BAP and 0.5–3.0 mg l^{-1} IAA resulted in indirect shoot regeneration. The shoots developed further on MS medium containing 0.5 mg l^{-1} BAP (6-benzylaminopurine) and 1.0 mg l^{-1} IAA, but high percentage of rooting was achieved only when 0.5 mg l^{-1} NAA and 1.0 mg l^{-1} IAA were used along with activated charcoal. The rooted plantlets showed healthy growth and survival upon transfer to jiffy pots containing sand, soil, and vermiculite in 1:1:1 ratio under poly house conditions. However, the exact survival percentage of plants was not mentioned. Later, the same group cultured germinated excised embryos on MS medium supplemented with 0.5–3.0 mg l^{-1} IAA, 0.5–2.0 mg l^{-1} NAA, 1.0–2.0 mg l^{-1} 2,4-D, 0.5–4.0 mg l^{-1} BAP, and 0.5–1.0 mg l^{-1} kinetin (Kn). The workers achieved maximum plantlet growth on MS medium supplemented with 1.0 mg l^{-1} IAA, 0.5 mg l^{-1} BA, and 1 % activated charcoal (Sultan et al. 2009). In the following year, Chakraborty et al. (2010) claimed the development of an efficient protocol for direct regeneration of *P. hexandrum* plants from rhizome explants. They reported the use of different PGR combinations, but the highest rate of multiple shoot formation was recorded on MS medium supplemented with 11.42 μM IAA and activated charcoal within 3 months from culture initiation. The next best combination was reported to be 2.68 μM NAA and 11.1 μM BAP. Rooting of in vitro shoots was reported only on half strength liquid MS medium containing 100 μM IBA (indole-3-butyric acid). However, when leaf explants were used, only callus formation but no shoot regeneration was reported. Guo et al. (2012) was the only group who used WPM or woody plant medium (Lloyd and McCown 1980) for shoot cultures followed by their rooting on medium containing 1.5 mg l^{-1} IAA and 0.5 mg l^{-1} NAA. When these shoots were transferred to jiffy pots containing turfy soil and perlite (2:1), 98.1 % survival was recorded. The plants were also reinstated in nature under open shady places at an altitude of 1700 m.

3.2.2 Root Cultures of *Podophyllum hexandrum*

Large-scale cultivation of adventitious roots offers a great opportunity for in vitro podophyllotoxins production. Roots derived from 2 months old in vitro raised seedlings were cultured on both B5 and MS media but higher growth with enhanced production of podophyllotoxins was achieved only on the former (Sagar and Zafar 2005). Similarly, Li et al. (2009) used mature embryos and rhizomes to establish root cultures on MS medium supplemented with different concentrations of IBA, GA_3, hydroquinone, and activated charcoal. Of these, only hydroquinone promoted increased length of rhizomes after 40 days, whereas activated charcoal hastened

optimal root biomass and podophyllotoxins accumulation. Another group, Rajesh et al. (2014c) developed adventitious roots from root segments and explored the possibilities of producing podophyllotoxins in them. The workers tested the effects of different parameters such as carbon sources, media strength, pH of initial medium, and the ratios of ammonium, nitrate, and phosphate in the culture medium. Finally, half strength MS medium containing 3.0 mg l^{-1} IBA with initial pH of 6.0 was optimized. The study also revealed that 2% sucrose was most effective in maximizing the biomass, whereas 6% sucrose was required for maximum podophyllotoxins accumulation. In addition, ammonium at 10 mM, nitrate at 20 mM, and phosphate at 2.25 mM were optimized. The optimized conditions led to a maximum podophyllotoxin accumulation up to 6.4 mg g^{-1}. However, 1.25 mM phosphate supported the highest growth.

3.2.3 Callus and Cell Cultures of *Podophyllum hexandrum* for the Production of Podophyllotoxins

Several studies have shown that secondary metabolites produced in tissue cultures are often higher than that in the parent plants. Hence, the technique became an attractive route for commercial-scale production of plant metabolites. Of the different techniques used in in vitro secondary metabolite production, suspension cultures being fast growing is considered to be the most effective. Moreover, it can also be manipulated easily.

In this regard, Uden et al. (1989) was the first person to report the production of podophyllotoxins in the cell cultures derived from root explants of in vitro raised plantlets. Suspension cultures were raised from callus developed on B5 medium supplemented with 2% coconut water, 4% sucrose, and 4.0 mg l^{-1} NAA. When these cultures were maintained under dark or light conditions at 26 °C, higher amounts of podophyllotoxins accumulation up to 0.3% on dry weight basis were recorded only in dark. In the following year, Uden et al. (1990) fed cell suspension cultures obtained from rhizomes with seven types of precursors such as phenylalanine, coniferin, tyrosine, cinnamic acid, caffeic acid, coumaric acid, and ferulic acid based on the phenylpropanoid pathway for enhanced production of podophyllotoxins. They also used a related compound, i.e., methylenedioxy cinnamic acid at 2.5 mM. Of these, only coniferin affected a 12.8-fold increase in podophyllotoxins. In a parallel work, Woerdenbag et al. (1990) used the same system for producing podophyllotoxins in cell suspension cultures obtained from root calli. However, when the cultures were fed with 3 mM of β-cyclodextrin-complexed coniferyl alcohol, higher accumulation of podophyllotoxins was recorded as in case of water-soluble coniferin (β-D-glucoside of coniferyl alcohol) fed cultures as compared to the poorly water-soluble noncomplexed coniferyl alcohol. In the same year, Heyenga et al. (1990) reported the production of tumor-inhibitory lignans in the callus cultures derived from root explants of in vitro raised seedlings. Callus was initiated and multiplied on B5 medium supplemented with 0.1–1.2 mg l^{-1} 2,4-D, 0.2–2.0 mg l^{-1} GA_3, and 0.05–1.0 mg l^{-1} BA. The cultures produced anticancerous lignans,

podophyllotoxin, 4′-demethylpodophyllotoxin, and podophyllotoxin-4-O-glucoside at levels similar to the original explants used for callus initiation. High levels of podophyllotoxins were recorded, particularly, when there was tissue differentiation. Moreover, the presence of plant growth regulators in the medium affected the relative proportions of podophyllotoxin and 4′-demethylpodophyllotoxin significantly.

In another study, Uden et al. (1995) developed cell cultures of *Linum flavum* and *P. hexandrum* for the bioconversion of cyclodextrin-complexed deoxypodophyllotoxin into podophyllotoxin and its 5-methoxy derivative. After developing callus cultures as per their earlier report of 1989, Uden et al. further studied the effect of cyclodextrin and its deoxypodophyllotoxin complex on the growth of cell suspension cultures. They found that the growth of the cultures was not affected by the presence of either of the compounds. Rather, maximum accumulation of podophyllotoxin and β-D-glucoside of podophyllotoxin (2.87 % on a dry weight basis) was recorded in deoxypodophyllotoxin-fed cultures after 9 days. This was the time when the highest bioconversion (33.2 %) occurred and corresponded with 192 mg l^{-1} of suspension cultures.

Chattopadhyay et al. (2001) developed suspension cultures on MS medium supplemented with 2.0 mg l^{-1} IAA, 10 g l^{-1} PVP, and 1.5 mg l^{-1} pectinase. PVP at 10 g l^{-1} was useful in eliminating problems such as browning of culture medium, clumping of cells, and reduction of pH in the medium. It also supported higher cell viability, biomass, and podophyllotoxin yield. Similarly, MS medium supplemented with 2.0 mg l^{-1} IAA and 5.0 g l^{-1} activated charcoal supported friable callus development. In the following year, the group used the same medium to develop calli from root explants after 3 weeks of culture at 20 °C (Chattopadhyay et al. 2002a). The workers also modulated the major media components such as carbon source, NH_4^+ to NO_3^- ratio, PO_4^-, and IAA for optimization of podophyllotoxin yield in the suspension cultures. They observed that glucose, inoculum, IAA, and pH were culture parameters that affected the podophyllotoxin yield significantly. However, production of podophyllotoxins was optimal only after the specific replacement of sucrose by glucose. Furthermore, the workers attempted submerged culturing in a 3 L stirred-tank bioreactor fitted with a low-shear, steric impeller for upscaling of podophyllotoxins (Chattopadhyay et al. 2002b). Podophyllotoxin accumulation was higher when the cells were gown in shake cultures at 100 rpm in dark. Upon screening B5, Eriksson, MS, Nitsch, Street, and White media, comparatively better growth and podophyllotoxin accumulation was supported on MS medium (Chattopadhyay et al. 2003a). Successful culturing in a 3 L stirred-tank bioreactor under low-shear condition in batch and fed batch modes of operation was also reported (Chattopadhyay et al. 2003b). Podophyllotoxin accumulation in batch mode was 21.4 g l^{-1} and 13.8 mg l^{-1} after 24 and 26 days, respectively. The nutrient feeding rate of 150 ml d^{-1} and substrate uptake rate of 105 g l^{-1} from incoming feed at non-limiting and non-inhibitory glucose concentrations were selected for cell retention bioreactors. After 60 days, there was an overall enhancement in the biomass (48.0 g l^{-1} dry cell weight) as well as podophyllotoxins (43.2 mg l^{-1}). However, when the bioreactor was optimized in the cell retention cultivated mode, the biomass and intracellular podophyllotoxin accumulation were 53.0 g l^{-1} and 48.8 mg l^{-1}, respectively

(Chattopadhyay et al. 2003c). When the podophyllotoxins produced in the cell cultures were studied for their cytotoxicity using human breast cancer cell line (MCF-7), there was 50 % inhibition by 1 nM podophyllotoxin, provided it was applied during the beginning of cell growth (Chattopadhyay et al. 2004).

Although other workers such as Ahmad et al. (2007) established callus cultures from excised embryo segments, no attempts were made by them to develop cell suspensions from these cultures. Majumder and Jha (2007, 2009) also established callus cultures from roots of in vitro raised seedlings but developed suspension cultures of only selected cell lines in B5 medium. When the cell lines were characterized using RAPD markers and podophyllotoxins, the podophyllotoxins content became stable after the fourth year of culture initiation.

Majumder (2012) also studied the effect of 100, 250, and 500 mg l^{-1} tryptophan, an indirect precursor of lignan biosynthesis on podophyllotoxins production in cell suspension cultures maintained in half strength liquid B5 medium containing 1 % (w/v) sucrose, 1.0 mg l^{-1} 2,4-D, 1.0 mg l^{-1} GA_3, and 0.1 mg l^{-1} BAP. Podophyllotoxin accumulation up to 2.7 times with no effect on biomass was observed as compared to control. In the same year, Bhattacharyya et al. (2012) initiated callus cultures from leaves in MS medium supplemented with 2.68 μM NAA and 8.88 μM BAP. However, cell suspension cultures developed in MS medium containing 60 mM nitrogen, 1.25 mM potassium dihydrogen phosphate, 6 % glucose, and 11.41 μM IAA. A seven to eight fold increase in podophyllotoxin accumulation was observed provided they were elicited with 100 μM methyl jasmonate after 9 days. MALDI TOF/TOF MS/MS analysis of the suspension cultures were also done to identify regulatory proteins.

In the present laboratory also, attempts were made to initiate callus cultures from leaves of plants growing in different locales of western Himalaya. MS medium containing 2 % sucrose, 5 μM IBA, and 10 μM BAP at pH 5.7 supported callus induction in leaves collected from Kukumseri region. After 1 month of callus induction, the medium had to be supplemented with 200 mg l^{-1} ascorbic acid to avoid polyphenol oxidation and browning of tissues. The calli proliferated further on the same medium (Fig. 3.1a–c). In contrast, the leaf explants collected from Parashar Lake at Mandi failed to respond. Hence, the response was considered to be region specific.

3.2.4 Secondary Metabolite Production Through *Agrobacterium*-Mediated Genetic Transformation of *Podophyllum hexandrum*

For the first time, Giri et al. (2001) attempted to enhance the production of podophyllotoxins through genetic transformation of *P. hexandrum*. The workers used the *Agrobacterium rhizogenes* strains A4, K599, and 15834 for transformation of callus derived from root explants. The transformed calli growing on half strength MS medium supplemented with 1.0 mg l^{-1} 2,4-D, 0.1 mg l^{-1} BAP, and 1.0 mg l^{-1} GA_3 showed a threefold increase in podophyllotoxins as compared to untransformed controls. Suspension cultures of the transformed calli were also initiated and

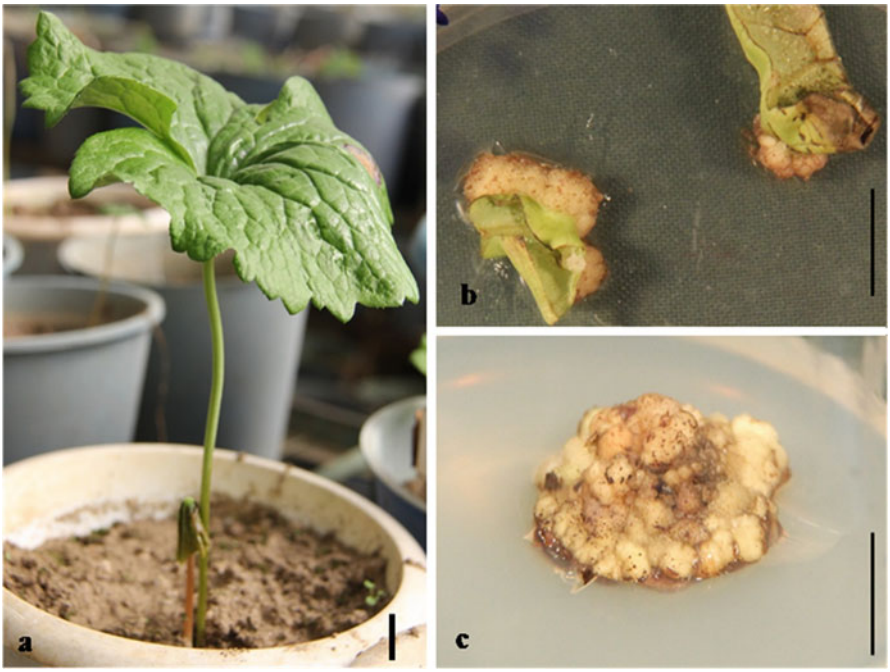

Fig. 3.1 Callusing in *P. hexandrum* plant collected from Kukumseri and maintained under polyhouse conditions, (**a**) 2-year-old plant, (**b**) initiation of callus on leaf segments, (**c**) callus proliferation. Bars = 1 cm

established on MS medium containing 1.0 mg l^{-1} 2,4-D and 0.1 mg l^{-1} BAP. In a separate strategy, Lin et al. (2003a, b) used the *A. rhizogenes* strains LBA9402 and TR105 for production of hairy roots from leaf disks of *Linum flavum*. Significant variation in coniferin accumulation was observed between hairy root lines originating from different *L. flavum* seedlings and/or *A. rhizogenes* strains. When roots were cultured on Linsmaier and Skoog (LS) medium with 2,4-D and NAA, coniferin accumulation was 58 mg g^{-1} dry weight. These hairy roots being a natural source of coniferin were further cocultured with *P. hexandrum* cell suspensions and maintained in dual shake flasks containing Linsmaier and Skoog (LS) medium in dual bioreactors. Increase in podophyllotoxins content was recorded. Earlier, availability and stability of coniferin in the medium were considered to be the key factors for podophyllotoxin synthesis during coculture. Therefore, podophyllotoxins in *P. hexandrum* cell suspensions was effectively increased through coniferin feeding (Lin et al. 2003a, b). Much later in 2013, Rajesh et al. used callus derived from zygotic embryos of *P. hexandrum* for *A. tumefaciens*-mediated genetic transformation. Different parameters governing genetic transformation such as acetosyringone concentration, time of cocultivation, and different strains of *A. tumefaciens* were tested. It was found that EHA105 harboring pCAMBIA2301 with *npt*II and *gus*A as selection marker and reporter genes, respectively, was the most effective as

compared to LBA4404 and EHA101 strains. While there was no effect of acetosyringone, the optimum time for cocultivation was 3 days on MS medium containing 1.5 mg l^{-1} 2,4-D. While complete elimination of residual *Agrobacterium* required the use of 200 mg l^{-1} timentin, kanamycin was used for the selection of the transformed somatic embryos. The putatively transformed plantlets were finally raised on basal MS medium and confirmed using GUS histochemical assay, PCR, and southern blotting. A transformation efficiency of 29.64 % was achieved.

3.3 *Picrorhiza kurroa*

3.3.1 In Vitro Regeneration

Clonal propagation of *P. kurroa* was first attempted by Lal et al. (1988) who used shoot tips for rapid proliferation of multiple shoots in basal MS medium supplemented with 3.0–5.0 mg l^{-1} Kn (kinetin). The workers found that medium containing 5.0 mg l^{-1} Kn evoked the best response of 50.6 ± 1.24 shoots per explant but concentrations beyond 7.0 mg l^{-1} promoted vitrification. Addition of 1.0 mg l^{-1} IAA improved shoot growth, leaf size, and stem thickness. Further, when the shoots were rooted and hardened in sterilized sand, soil, and manure at 1:1:1 ratio, survival of 87.7 % plants was recorded. Thereafter, several workers attempted micropropagation of *P. kurroa* and different explants were used by them (Mondal et al. 2013). Upadhyay et al. (1989) cultured terminal and single nodes on MS medium containing 0.11–2.25 mg l^{-1} BAP and 0.02–0.2 mg l^{-1} IAA or 0.03–0.35 mg l^{-1} GA_3. Of these, only 0.2 mg l^{-1} BAP was most effective. Again in 1996, Lal and Ahuja induced callusing on leaf explants by using MS medium containing 2.0 mg l^{-1} 2,4-D but supplementation of 4.0 mg l^{-1} NAA and 1.0 mg l^{-1} Kn was required for further growth and proliferation. Induction of shoot bud primordia occurred only when the calli were transferred to MS medium containing 0.25 mg l^{-1} BAP. Shoot growth and multiplication was however supported when nitrogen in MS medium was reduced to half strength and 0.12 mg l^{-1} BAP was added. Another group used axillary shoot buds and cotyledonary nodes of seedlings for shoot multiplication on MS medium containing 1.0 μM BAP or Kn (Chandra et al. 2004, 2006). For hardening the plants, the rooted shoots were transferred to a mixture of soil and sand at 2:1 ratio and also fortified with *Bacillus subtilis* and *Pseudomonas corrugate*. As a result, they achieved 92.5 and 85.0 % plantlet survival, respectively, after 2 months. However, when the same group hardened the plantlets in soil and sand (1:1) under high relative humidity (80.0 ± 5.0 %), only 65 % survival was recorded after 6 months (Chandra et al. 2006). Later in 2009a, Sood and Chauhan cultured axillary shoot tips on medium containing both Kn and IBA for shoot multiplication. The workers also developed a low-cost medium by replacing sucrose with table sugar and omitting agar-agar completely. In an attempt to develop a protocol for large-scale propagation of *P. kurroa*, liquid MS medium supplemented with 2.0 mg l^{-1} IBA, 3.0 mg l^{-1} Kn, and 3 % table sugar was tested (Sood and Chauhan 2009a). However, the number of shoots per explant was reduced to 27 only. The workers also cultured leaf

disks, nodal, and root segments on MS medium supplemented with 2.0 mg l^{-1} 2,4-D and 0.5 mg l^{-1} IBA and achieved callusing in 56.3, 38.8, and 70 % explants, respectively. Multiple shoot formation on these calli was also recorded on MS medium containing 0.5–2.0 mg l^{-1} BAP, 1.0–3.0 mg l^{-1} Kn, and 0.5–2.0 mg l^{-1} IBA. Of these, 2.0 mg l^{-1} BAP and 3.0 mg l^{-1} Kn was reported to be the best for shoot multiplication (Sood and Chauhan 2009b). The workers also hardened the rooted shoots in sterile mixture of sand, soil, and vermiculite (1:1:1). In the following year, Jan et al. (2010) optimized the process of indirect shoot regeneration from nodal explants. Although different concentrations of 2,4-D and BAP were used in MS medium, only 0.25 mg l^{-1} each of 2,4-D and BAP supported callus induction in 20 % explants. Indirect shoot regeneration followed by their elongation and multiplication was however evoked only when 0.2–1.0 mg l^{-1} NAA was used along with 0.1–0.5 mg l^{-1} each of IAA and IBA. As a result, there was a maximum of 18.5 ± 1.10 shoots per explant on 0.2 mg l^{-1} NAA as compared to 18.3 ± 0.57 shoots per explant in 0.6 mg l^{-1} NAA in 80 and 95 % of explants, respectively. As in case of earlier report, the workers used autoclaved mixture of sand, soil, and vermiculite at 1:1:1 ratio for hardening of rooted shoots and achieved 81.5 % plantlet survival. In the same year, Sharma et al. (2010) employed 2.0 mg l^{-1} Kn for shoot proliferation from leaf explants, axillary buds, and nodal explants. Maximum shoot regeneration was achieved by these workers on medium containing 2.0 mg l^{-1} Kn alone as well as in combination with 0.50 mg l^{-1} IBA. The workers also considered "synthetic seeds" as an effective system for ex situ conservation of *P. kurroa* and their large-scale plantations in degraded habitats (Sharma et al. 2010). However, the usefulness of the "synthetic seeds technique" was actually demonstrated by Mishra et al. (2011a). Hence, the workers developed alginate beads of somatic embryos and in vitro-grown shoots of *P. kurroa*. They also evaluated their revival potential after different durations of storage. The encapsulated micro-shoots showed 89.33 % revival after 3 months of storage at 4 °C with 42.66 % multiple shoot formation as well as 21.43 % shoot and root formation. However, transfer to half strength MS medium containing 0.2 mg l^{-1} NAA was necessary for healthy root formation. When these plantlets were transferred to greenhouse conditions, their survival was 95 %. Another group, i.e., Patial and coworkers, showed the benefits of 15-day pulse treatment of TDZ. The researchers used leaf explants from in vitro raised plants (derived from the leaves of field grown plants). They found that when the abaxial surfaces of leaf segments touched the culture medium (i.e., MS containing 2.32 μM Kn), there was maximum regeneration of indirect shoots (42.0) from the middle portion of about 94 % explants. Direct regeneration of shoot buds on plant growth regulator-free MS medium was also recorded. Shoots obtained either via direct or indirect method were further multiplied on MS medium containing 2.32 μM Kn (Patial et al. 2012) (Fig. 3.2a–g). Later, Sharma et al. (2015) attempted to culture 0.5–1.0 cm long shoot apices on MS medium supplemented with seaweed extracts. After 1 month, several fold enhancements in total biomass, length, and number of shoots as well as roots was recorded as compared to control. The plants also showed 80 % survival under greenhouse conditions.

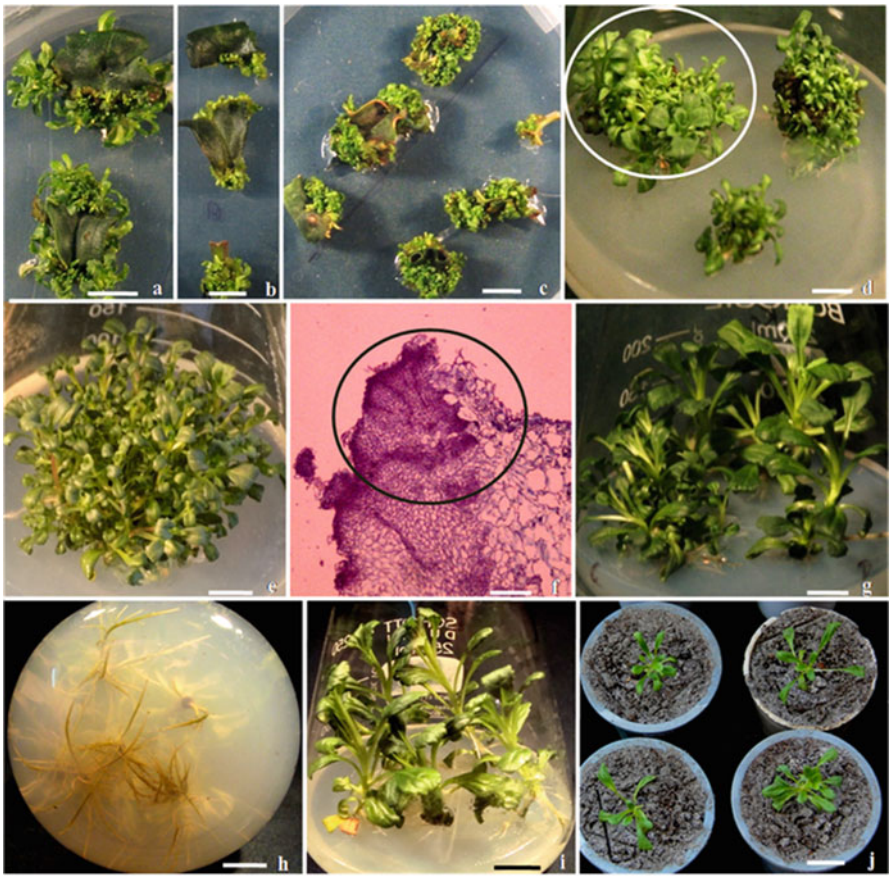

Fig. 3.2 Shoot regeneration from leaf explants of *P. kurroa* (**a**) shoot bud regeneration from ex vitro leaves, (**b**) direct shoot bud initiation from leaves of in vitro raised plant, (**c**) regeneration response of abaxial (on *left side*) and adaxial (on *right side*) surfaces of leaf explant, (**d**–**e**) maximum regeneration from middle portion of leaf explant (*encircled*), (**f**) histological section of leaf callus showing emergence of shoot bud (*encircled*), (**g**) shoot multiplication on MS medium containing Kn, (**h**) root formation on MS basal medium, (**i**) shoots at 15 °C, (**j**) hardened plants in polyhouse. Bars = 1 cm

Overall, various explants such as shoot tips, nodal segments, roots, and even leaf explants were used by researchers working on in vitro regeneration of *P. kurroa*. The percentage usage of these explants was found to range between 12 and 23 % (Fig. 3.3).

The step involving acclimatization of tissue culture-raised plants is crucial in governing the success of plant conservation and improvement. In this regard, hardening of tissue culture-raised *P. kurroa* plants has been a serious problem because of high percentage of plant mortality even after months of active and healthy growth. This is because of their high susceptibility to fungal diseases that cause rotting of

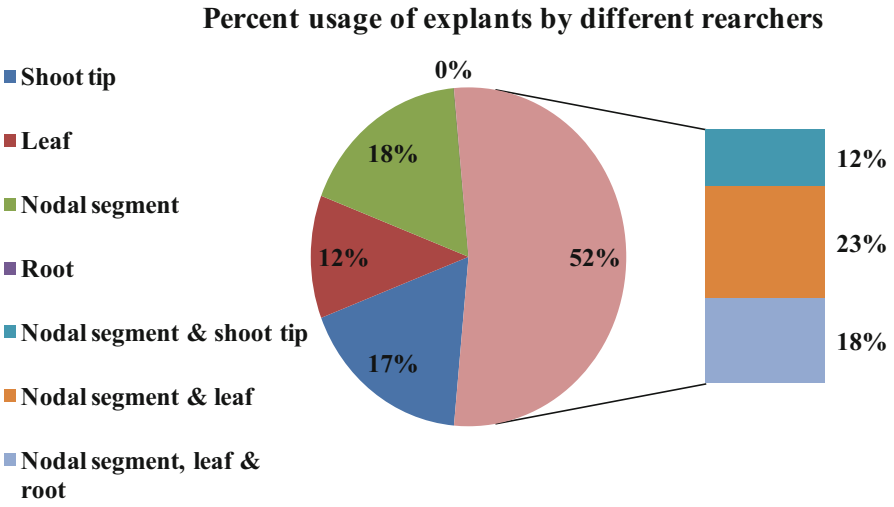

Fig. 3.3 Percent usage of explants by different researchers for initiation of aseptic cultures of *P. kurroa*

the aerial and underground parts. Therefore, different workers attempted biological hardening of tissue culture-raised plants of *P. kurroa*. Trivedi and Pandey (2007) used *Bacillus megaterium*, *B. subtilis*, and *Pseudomonas corrugate* for hardening in vitro raised shoots after rooting on MS medium supplemented with 0.22 mg l^{-1} IBA. The plants maintained for 8 weeks under greenhouse conditions at >80 % humidity showed 94 % survival as compared to control (38.5 %) upon transfer to larger pots. Patial et al. (2012), on the other hand, cultured rooted shoots (derived indirectly from leaf explants) at 15 °C for 10 days and achieved higher survival of the plants (80 %) as compared to controls (50.0 %) kept at 25 °C (Fig. 3.2g–j). The treated plants were found to have healthier leaves with thick cuticles and well-differentiated palisade and spongy parenchyma. These attributes were considered responsible for helping the plants to cope with stress imposed by the hardening process. In the following year, Thakur et al. (2013) attempted to optimize the root yield of *P. kurroa* by treating the plants with biofertilizers singly or in combination with farm yard manure and/or vermicompost. Farm yard manure or vermicompost in combination with *Azotobacter*, a phosphate-solubilizing bacteria (strain B or F), and vascular-arbuscular mycorrhizae resulted in 947 kg ha^{-1} root yield in 3-year-old plants. The fungal endophyte *Piriformospora indica* was also used for biological hardening (Das et al. 2015) and a 1.3-fold increase in the survival of tissue culture-raised plants was recorded under greenhouse conditions. Recently, Helena et al. (2015) developed an indirect system of regeneration from leaf and stem segments. MS medium containing 0.5 mg l^{-1} TDZ (thidiazuron) in combination with 0.3 and 0.5 mg l^{-1} IBA evoked callus induction from leaves and stem segments, respectively. However, shoot regeneration of about 89 % was evoked on MS medium containing 1.0 mg l^{-1} BA and 0.75 mg l^{-1} Kn in case of leaves, whereas 1.0 mg l^{-1} BA and

1.0 mg l^{-1} Kn for stem segments. When these shoots were transferred to half strength MS medium containing 0.5 mg l^{-1} 2–4, D and 0.4 mg l^{-1}NAA, there was 100 % root induction.

3.3.2 Genetic Transformation

Hairy root cultures of *P. kurroa* from leaf and stem explants was first reported by Verma et al. (2007). The researchers obtained 66.7 % relative transformation frequency after 3 weeks of transformation with *Agrobacterium rhizogenes* strain, LBA9402. They also evaluated nine independent opine and TL-positive hairy root somaclones or rhizoclones for their ability to produce kutkoside and picroside I during different phases of growth. Based on the inter-clonal variations in the contents of these compounds in the rhizoclones, the hairy root rhizoclone 14-P was selected for the highest biomass and kutkoside and picroside I contents. Four years later, Mishra et al. (2011b) used the *A. rhizogenes* strains, A4, and PAT405 for induction of hairy root cultures from leaf, internodal segments, and shoot tips. The hairy roots were evaluated after 8 weeks. The A4 strain was found to yield higher contents of both picrotin and picrotoxinin (8.8 and 47.1 μg l^{-1} on dry weight basis, respectively). In contrast, the PAT405 strain yielded 4.45 μg l^{-1} picrotin on dry weight basis as compared untransformed control (0.64 μg l^{-1} picrotin on dry weight basis). In the following year, Praveena and Rao (2012) employed *Agrobacterium rhizogenes*-mediated transformation as well as physical (UV radiation) and chemical (acridine dyes) mutagenesis approach for enhanced production of picrosides and kutkosides in in vitro roots. The kutkin content in the transformed roots was the highest (0.62 μg ml^{-1}) as compared to control (0.53 μg ml^{-1}) or roots subjected to UV radiation (0.58 μg ml^{-1}) and acridine dyes (0.24 μg ml^{-1}). In the same year, Bhat et al. (2012) attempted to transform the leaf explants of *P. kurroa* with the binary vector pCAMBIA1302 harboring the hygromycin phosphotransferase and green fluorescent protein (*gfp*) encoding genes in the strain GV3101 of *A. tumefaciens*. Their study revealed that in vitro-grown explants pre-cultured for 2 days on regeneration medium prior to cocultivation in presence of 200 μM acetosyringone was the most effective. Finally, putative transformants selected on 15 mg l^{-1} hygromycin were found to test positive in PCR (56 %) and also showed *gfp* expression in fluorescence microscopy.

3.4 Conclusions

In conclusion, the review is a compiled information on various in vitro approaches employed for the conservation and secondary metabolites production in *Podophyllum hexandrum* and *Picrorhiza kurroa*. The article offers a base line information from which various gaps can be identified and addressed for future research in these endangered medicinal herbs of western Himalaya.

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