2 Selection of Elites and In Vitro Propagation of Selected High-Value Himalayan Medicinal Herbs for Sustainable Utilization and Conservation

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Abstract

Reduction in the forest cover from the Indian Himalayan region (IHR), due to overexploitation, has resulted in decreased availability of non-timber forest products, including medicinal plants of high economic value. With the ever-increasing human population and growing demand for plants and plant-derived products,

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M. Anis, N. Ahmad (eds.), *Plant Tissue Culture: Propagation, Conservation and Crop Improvement*, DOI 10.1007/978-981-10-1917-3_2

there has been tremendous anthropogenic pressure on these primary producers. Many plant species are a source of high-value drugs; due to increasing global demand for the 'naturals', they are being subjected to reckless, often illegal harvesting, well beyond the natural regeneration capacity. This has led to many species being listed in the Red Data Book or in various IUCN threat categories. Improper harvesting (season and/or age of the plant/plant parts) not only results in uneconomical yields due to low content of active ingredients but also adversely affects the process of natural regeneration. There is, therefore, an urgent need for commercially important species to be subjected to improved management practices and regulated harvesting to generate better economic benefits on one hand and to encourage cultivation for sustained utilization as well as economic development of the region on the other. This twin strategy would also help to improve the conservation status of such species.

In order to meet such challenges, in vitro propagation (tissue culture) techniques have provided a well-recognized potential for rapid multiplication of elite clones for the supply of much needed good-quality planting material for cultivation and also to achieve conservation objectives. Keeping these goals in mind, studies were taken up to assess the active ingredient content of plants/plant parts collected from natural populations growing in different locations/altitudes in the wild and to develop in vitro propagation methods for selected high-value alpine medicinal herbs (*Aconitum balfourii*, *A. heterophyllum*, *Picrorhiza kurrooa* and *Podophyllum hexandrum*). Using elite plant material, attempts have been made to establish tissue culture protocols that involved the induction of multiple shoots, improved rooting and subsequent development of suitable methods for hardening and field transfer. In a few cases, the survival and growth of tissue culture-raised (TCR) plants was also monitored to evaluate their field performance.

Abbreviations

BAP	6-Benzylaminopurine
IBA	Indole-3-butyric acid
IAA	Indole-3-acetic acid
IHR	Indian Himalayan region
GA_3	Gibberellic acid
Kn	Kinetin
MS	Murashige and Skoog
NAA	α-Naphthalene acetic acid
PGS	Plant growth substance
SR	Seed raised
TCR	Tissue culture raised
TDZ	(Thidiazuron): 1-phenyl-3 (1,2,3-thiadiazol-5-yl) urea

2.1 Introduction

The forest cover from the Indian Himalayan region (IHR) has been substantially reduced over the years, and it varies from 10.14% (Jammu and Kashmir) to 90.38% (Mizoram) across IHR states (Anonymous 2013). The recommended cover of 67% and above is not present in many of the Himalayan states. This has adversely affected the availability of non-timber forest products, including medicinal plants of high commercial and therapeutic value. The increasing human population and the growing demand for plants and plant-based products have collectively placed very high anthropogenic pressure on these primary producers. Many plant species are known sources of high-value drugs, and due to the increasing global demand for the 'naturals', they are being subjected to reckless, often illegal harvesting, well beyond their natural regenerative capacity. This has led to many species being listed in the Red Data Book and/or in various threat categories (Nandi et al. 2002; Anonymous 2003; Ved et al. 2003).

The life and economy of the hill people, to a large extent, depend on the plants, and thus any reduction in the forest cover does have a great negative effect on natural resources including their living conditions. Moreover, improper harvesting (season and/or age of the plant/plant parts) results in uneconomical yields due to the suboptimal content of active ingredients and also adversely affects the process of natural regeneration. There is, therefore, an urgent need for all such commercially important species to be subjected to improved management practices and regulated harvesting to generate improved long-term economic benefits on one hand and to encourage their cultivation for sustained utilization as well as economic development of the region on the other. Keeping these goals in mind, studies were taken up to assess the active ingredient content of plants/plant parts collected from natural populations of selected medicinal plants growing in different locations/altitudes in the wild and to develop in vitro propagation (tissue culture based) methods for these high-value alpine medicinal herbs (*Aconitum balfourii*, *A. heterophyllum*, *Picrorhiza kurrooa* and *Podophyllum hexandrum*). Using elite (in terms of high active principle content) plant material, attempts have been made to establish their in vitro (tissue) cultures, induce multiple shoots, improve rooting of shoots and subsequently develop suitable methods for hardening before field transfer. In a few cases, the survival and growth of in vitro-raised (IVR) plants was also monitored to evaluate field performance.

A brief description of all four species selected (Fig. 2.1) for in vitro propagation has been provided below.

Aconitum balfourii Stapf. [=*A. atrox* (Brhul) Muk.; family, Ranunculaceae; English name, aconite; local names, 'Meetha' and 'Bish'] is a highly valued medicinal herb endemic to the alpine and subalpine belts of the IHR and grows above 3200 m altitude (Samant et al. 1998). Its tuberous roots are used by various ethnic communities for curing different ailments (rheumatism, fever, etc.) and are important source of ingredients used in the preparation of Indian Ayurvedic medicines (Chopra et al. 1984; Anonymous 1988). The medicinal properties have been attributed to several diterpenoid alkaloids, mainly aconitine, balfourine, bikhaconitine and pseudaconitine, the latter being highly toxic and biologically 2.5 times more active than aconitine (Chopra et al. 1984; Khetwal et al. 1992).

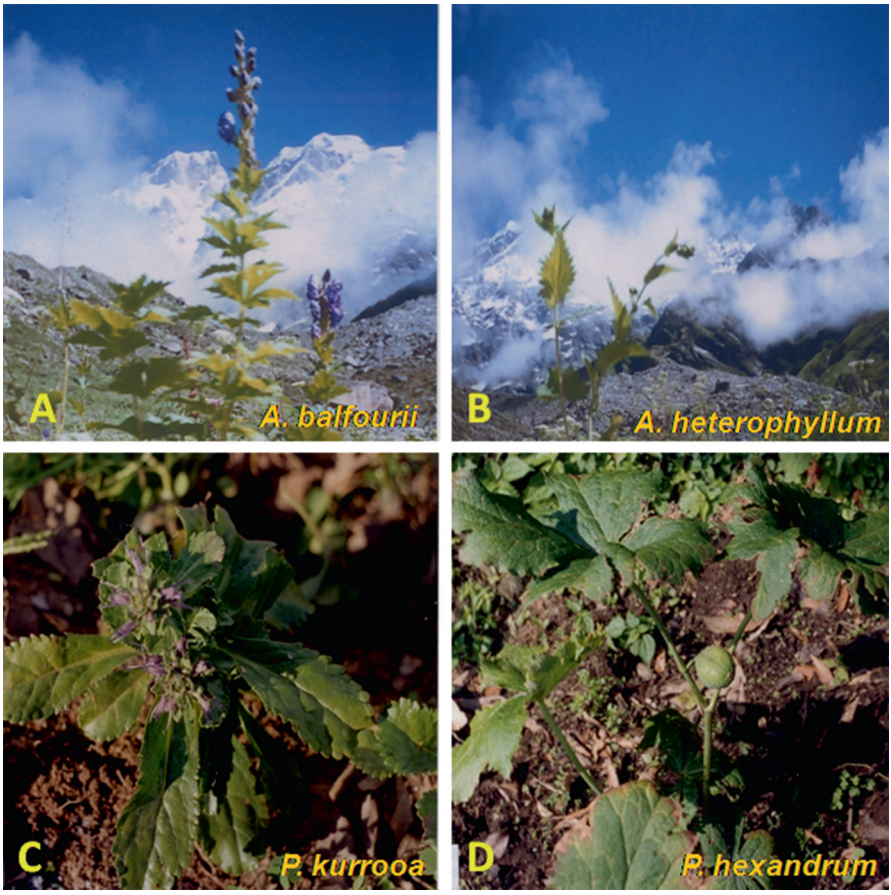

Fig. 2.1 Different medicinal plants used in the study growing under the natural habitat. (**a**) *Aconitum balfourii*, (**b**) *A. heterophyllum*, (**c**) *Picrorhiza kurrooa* and (**d**) *Podophyllum hexandrum*

Aconitum heterophyllum Wall. (family, Ranunculaceae; common name, aconite; local name, 'Atish') is an erect herbaceous rosette distributed in the subalpine and alpine regions of IHR at 3000 m and above. The tubers and roots are being used as tonic and for curing different ailments like fever, diarrhoea and dyspepsia. The tubers and roots are known to contain alkaloids like atisine, heteratisine and aconitine (Pelletier et al. 1968; Pandey et al. 2008). Both *A. balfourii* and *A. heterophyllum* pass through long juvenile phase and their propagation, mainly through seeds, is quite poor under natural conditions. Flowering and fruiting are erratic due to harsh climatic conditions, and only limited or no viable seeds are produced (Pandey et al. 2000 and references therein). At present *A. balfourii* and *A. heterophyllum* are under 'vulnerable' and 'critically endangered' category, respectively (Ved et al. 2003).

Picrorhiza kurrooa Royle ex Benth. (family: Scrophulariaceae; local name, 'Kutki') is endemic to Himalayan alpines and distributed between 3300 and 4800 m

altitude. The extracts of runners and roots have been used in several 'Ayurvedic' preparations, prescribed for hepatic disorders. The plant is also used in traditional as well as modern system of medicine as a stomachic, purgative, antiperiodic and brain tonic and in dyspepsia and fever. It contains picroside I, II and III and kutkoside as major bioactive compounds (Weinges et al. 1972; Jia et al. 1999). Indiscriminate collection of this plant from the wilds and lack of organized cultivation have led to considerable depletion of its natural populations, and it is presently categorized as 'critically endangered' and listed in CITES Appendix II (Anonymous 2003; Ved et al. 2003).

Podophyllum hexandrum (family, Podophyllaceae; English name, mayapple; Hindi name, 'Bankakri'; local names, 'Banwaigan' and 'Papri') has gained considerable importance because the rhizomes of this species (and that of some other *Podophyllum* species) contain several lignans, out of which one of the non-alkaloid compounds, podophyllotoxin, is extremely important (Van Uden et al. 1989; Canel et al. 2000; Tabassum et al. 2014). It is a potent antiviral agent (Beutner and von Krogh 1990) as well as the starting compound for the preparation of semisynthetic compounds, namely, etoposide, Etopophos and teniposide, which are effective antitumour agents used in the treatment of lung cancer, variety of leukaemia and other solid tumours (Van Uden et al. 1989; Canel et al. 2000; Lee and Xiao 2005). Two species, namely, *P. hexandrum* Royle (occurring in Central Himalaya) and *P. sikkimensis* R. Chatterji and Mukerji (found in Eastern Himalaya) have been reported from India. *P. hexandrum* (generally growing above 2800 m) contains three times more podophyllotoxin as compared to the American species, *P. peltatum*, and hence its ever-increasing demand. Its uncontrolled collection from the natural populations is inflicting tremendous damage; *P. hexandrum* is currently placed under 'endangered' category and listed in CITES Appendix II (Anonymous 2003; Ved et al. 2003). Details of these three species can be found in a recent article (Paul et al. 2013).

2.2 Analysis of Active Ingredients

2.2.1 Aconitine and Pseudaconitine

Quantification of diterpenoid alkaloids, namely, aconitine and pseudaconitine, was carried out in tubers of *Aconitum heterophyllum* and *A. balfourii* collected from higher altitudes of Indian Central Himalaya [seven locations (3000–3600 m) in Garhwal and four locations (3250–3430 m) in Kumaun region of Uttarakhand] following column, thin-layer and high-performance liquid chromatography (HPLC) with the aim of identifying elites (Pandey et al. 2008). The aconitine levels in different populations of *A. heterophyllum* varied from 0.13 to 0.75 % (dry weight basis); maximum and minimum levels were detected in tubers from Phurkia (3260 m) and Kafni (3400 m), respectively. In *A. balfourii* the amount of aconitine and pseudaconitine also varied and was found to range from 0.13–0.83 % to 0.06–0.62 %, respectively. The highest level of pseudaconitine (0.62 %) was recorded in samples collected from Phurkia Bugyal (3430 m), while the lowest and about tenfold less value (0.06 %) was recorded in tubers from Kafni population (3400 m);

highest level of aconitine (0.83 %) was also recorded in tubers collected from Phurkia Bugyal (3430 m), while the lowest values were found in samples from Kedarnath population (3600 m). The active principle content could not be correlated with altitude (Pandey et al. 2008). However, in another study, the amount of pseudaconitine and aconitine content in *A. balfourii* and *A. heterophyllum* was found to generally increase with altitude (Bahuguna et al. 2000). The amount of pseudaconitine and aconitine reported by Pandey et al. (2008) was higher than the values reported for these species by Bahuguna et al. (2000). The existing natural populations are likely to be of seed origin and thus the observed variation in active ingredient content could be attributed to genotypic differences. Ecological factors like habitat, temperature and soil characteristics are likely to affect qualitative and quantitative changes in aconitine analogues; reported variation in the levels of active principles in different studies could also result from the methods used for the extraction, purification and estimation.

A number of important chemical compounds identified from various *Aconitum* species along with their biological activities have been summarized in recent reviews (Srivastava et al. 2010; Sharma and Gaur 2012).

2.2.2 Picrosides

The runners/stolons of *P. kurrooa* are known to contain different medicinal and bioactive compounds which act as hepatoprotective agents and been identified as picroside I, picroside II, kutkoside, apocynin, androsin, cucurbitacin glycosides, catechol, etc. (Weinges et al. 1972; Jia et al. 1999). *P. scrophulariiflora* is also a source of iridoid glycosides such as picroside I, picroside II and kutkoside; however, it contains an additional phenylethanoid glycoside and plantamajoside which are absent in *P. kurrooa* (Li et al. 1998), and hence *P. scrophulariiflora* is a better substitute for *P. kurrooa*. Kutkoside was thought to be a single compound; however a recent report indicates that kutkoside is a mixture of several iridoid glycosides, namely, picroside II, picroside IV and 6-ferulloylcatalpol (Bhandari et al. 2010). Extracts of both *P. kurrooa* and *P. scrophulariiflora* have been reported to contain high antioxidant activity (Ray et al. 2002; Bhandari et al. 2010; Tiwari et al. 2012).

There are limited reports on active ingredient content of runner/stolon of *P. kurrooa* collected from different populations. Purohit et al. (2008) analyzed five high-altitude populations (narrow and broadleaf plants, collected from Tungnath, Kilpur, Valley of Flowers, Kuwari Pass and Panwali Kantha at 2700–3800 m altitudes) from Garhwal Himalaya in Uttarakhand and reported that picrotin and picrotoxin content ranged from 1.00 to 6.05 mg/g. In general, the broadleaf plants from all the populations showed higher content than the narrow ones. The minimum (1.0 mg/g) picrotin and picrotoxin content was found in narrow leaf plant samples from Kuwari Pass (2800–3800 m) population, while the maximum amount (6.05 mg/g) was reported from broadleaf samples from Valley of Flowers (2700–3600 m) population (Purohit et al. 2008). Sharma et al. (2012b) analyzed runner samples from seven accessions of *P. kurrooa* collected from high-altitude regions of Himachal Pradesh, India (e.g.

Chamba, Manikaran, Manali, Khoksar, Marhi, Keylong and Rohtang from 996 to 3978 m altitude) by reversed-phase HPLC and reported that the highest level of the major bioactive compounds, picroside I (3.5 %) and picroside II (2.0 %), was found in Rohtang population (3978 m). The variability of these major constituents within the same species at different altitudinal ranges would thus help in identifying superior clones (elites) for multiplication and conservation.

In another study (Tiwari et al. 2012) picroside content and antioxidant activity were determined in rhizomes of *P. scrophulariiflora* and *P. kurrooa* collected from Munsyari region of Uttarakhand, India. Separation and quantification was achieved by HPTLC and subsequently by densitometrically. The study revealed that picroside I and picroside II content was higher in *P. scrophulariiflora* than in *P. kurrooa*. Picroside I content was found to be 1.26 and 1.61 %, and picroside II was 0.48 and 0.61 % in *P. kurrooa* and *P. scrophulariiflora*, respectively. The antioxidant potential of these two species using DPPH assay was found to be quite high, i.e. at a concentration of 0.1 mg/ml, the scavenging activities of *P. kurrooa* and *P. scrophulariiflora* were found to be 37.70 and 34.30 %, respectively (Tiwari et al. 2012). In another investigation, high levels of picrosides (picroside I and picroside II, up to 7.33 %) were reported in rhizomes collected from one of the three different populations of *P. scrophulariiflora* from the Eastern Himalayan region (Bantawa et al. 2010). These workers also reported a micropropagation protocol for rapid multiplication of this high picroside containing population which can be used for cultivation and conservation.

2.2.3 Podophyllotoxin

Podophyllotoxin levels were determined in rhizome and root samples of *P. hexandrum* plants (with known leaf morphological variants, i.e. 1 L, 2 L and 3 L; 4 L samples could not be obtained) collected from 17 different populations (2800–3600 m altitudes) along an altitudinal gradient spread across Uttarakhand state (Pandey 2002; Pandey et al. 2015). Extraction, purification and subsequent analysis (by HPLC) were carried out by published methods (Van Uden et al. 1989; Nadeem et al. 2007), and the results indicated a wide variation in the podophyllotoxin content, ranging from 0.012 to 5.80 % (on dry weight basis); among these morphological variants, 2 L plants of Kedarnath area (highest altitude, 3600 m) exhibited maximum content, both in rhizomes and roots (Pandey 2002; Pandey et al. 2015). In another investigation, analyses of rhizomes collected from eight populations along an altitudinal gradient (2740–3350 m, i.e. Dhungiadhaung, 2740 m; Dwali, 2790 m; Juharpani, 2900 m; Khamia, 3125 m; Khatia, 3240 m; Kathlia I, 3250 m; Phurkia, 3260 m; and Kathlia II. 3350 m) of Kumaun region in Indian Central Himalaya indicated variation in podophyllotoxin levels, ranging from 0.36 % to 1.08 % (Nadeem et al. 2007). It was observed that the minimum podophyllotoxin content (0.36 %) was recorded in samples from Dwali (2790 m), while the maximum (1.08 %) was detected in samples from Kathlia II (3260 m) populations; the regression analysis revealed a positive correlation between podophyllotoxin content and increase in the altitude (Nadeem et al. 2007).

Podophyllotoxin levels in rhizomes have been reported to vary considerably, and values up to 8.26 % were found in samples collected from the states of Uttarakhand and Himachal Pradesh in IHR (Purohit et al. 1998, 1999; Sharma et al. 2000). Furthermore, levels ranging from 3.02 to 9.53 % were reported from 28 populations occurring at various altitudes (1570–4300 m) in Himachal Pradesh (Naik et al. 2010). Similar levels were also estimated from the rhizome buds of various populations collected from Zanskar valley of Jammu and Kashmir state in IHR (Kitchlu et al. 2011). Sharma (2013) has also reported podophyllotoxin levels ranging from 3.44 to 5.87 % in rhizomes collected from Himachal Pradesh, but no relationship between the active principle content and altitude was observed.

Besides rhizomes, leaves of *P. peltatum* have also been reported to be a rich source of podophyllotoxin (Bastos et al. 1996; Canel et al. 2001; Moraes et al. 2000, 2002). However, the occurrence of podophyllotoxin in leaves of *P. hexandrum* from wild has been reported only recently (Pandey et al. 2013; Sharma 2013). In a detailed study, podophyllotoxin content in leaf and stem samples of *P. hexandrum* plants (with leaf morphological variants, i.e. 1 L, 2 L and 3 L) collected from seven different populations (2800–3600 m) of Uttarakhand was analyzed by established methods, and the content was found to range from 0.001 to 0.60 %; among these morphological variants, 3 L plants of Dodital area (altitude, 3100 m) exhibited maximum content, both in leaf and stem samples (Pandey et al. 2013). However, these estimates were found to be lower as compared to values up to 5.80 % reported from rhizomes/roots collected from the same region (Pandey 2002, Pandey et al. 2015). Sharma (2013) also determined the podophyllotoxin content of *P. hexandrum* leaf samples from four high-altitude populations (2730–3978 m, Himachal Pradesh), and the maximum amount (0.30 %) was found in Marhi (3300 m) population. The podophyllotoxin content of leaves was nearly two-fold higher (0.60 %) in a study conducted by Pandey et al. (2013) as compared to the report by Sharma (2013). On the other hand, levels of up to 5.2 % have been reported, both in leaves and rhizomes of *P. peltatum* (Bastos et al. 1996; Canel et al. 2001; Moraes et al. 2000, 2002, 2005; Cushman et al. 2005; Zheljazkov et al. 2011). Thus leaves can be an alternate and readily renewable source of the compound which is routinely in high demand by pharmaceutical industries.

It is important to mention that in all above cited studies, a wide variation in podophyllotoxin content was observed. Thus, the observed differences in content in various populations of *P. peltatum* and *P. hexandrum* can be ascribed to genotypic differences (Bastos et al. 1996; Moraes et al. 2000; Nadeem et al. 2007; Naik et al. 2010; Pandey et al. 2013). Further, the age of the plant also influences active ingredient content in rhizomes of *P. hexandrum* (Pandey et al. 2007; Sharma et al. 2000). In this investigation analyses were carried out on samples from wild populations; thus, the age of sampled plants could not be ascertained. It is possible that variations arise due to the presence of different chemo types in natural populations as also on the method of extraction (Bastos et al. 1996; Canel et al. 2001). Both biotic and abiotic factors, including soil conditions, are known to affect the lignan yield in *P. peltatum* (Moraes et al. 2005).

Table 2.1 Podophyllotoxin content in different parts of *P. hexandrum* plants (1- to 5-year-old, seed raised) grown at a lower altitude (Kosi, 1150 m)

	Podophyllotoxin content (% of dry wt)		
Plant age (year)	Rhizome	Roots	Leaf
1	0.016 ± 0.001	0.022 ± 0.008	0.005 ± 0.001
2	0.017 ± 0.002	0.039 ± 0.003	0.009 ± 0.003
3	0.040 ± 0.001	0.056 ± 0.000	ND
4	0.080 ± 0.021	0.159 ± 0.012	ND
5	0.108 ± 0.026	0.011 ± 0.005	0.0003 ± 0.0001
LSD ($P = 0.005$)	0.056	0.003	0.007

Source: Pandey et al. (2007)
LSD Least significant difference, ± = Standard error of mean; Values are an average of three determinations. *ND* Not detected

An attempt was also made to grow plants of *P. hexandrum* at a lower altitude (1150 m altitude) in order to examine if there are changes in podophyllotoxin content in plants of known age; such study should also help in suggesting a suitable strategy for promoting cultivation at alternate locations to reduce the pressure on natural populations and for developing cultivation packages. The podophyllotoxin content in various plant parts (rhizomes, roots and leaves) of *P. hexandrum* (expressed as % of dry wt) is summarized (Table 2.1, Pandey et al. 2007). The content in rhizomes was low and similar for 1- and 2-year-old plants and increased gradually reaching a maximum value (0.108 %) in 5-year-old plants. In general, it was higher in roots than in the rhizomes of 1- to 4-year-old plants. The content in the roots increased up to 4 years when highest level (0.159 %) of podophyllotoxin was recorded; this level was higher than that present in rhizomes of 5-year-old plants. However, the levels in the roots declined drastically in 5-year-old plants. The podophyllotoxin content of leaves was considerably lower than that of rhizomes and roots of the same plants and was found in the range of 0.0003–0.009 %; the levels were below detectable limit in 3- and 4-year-old plants. It must be added that various growth parameters (leaf area, leaf length and above- and below-ground biomass) recorded a concomitant increase with plant age (Pandey et al. 2007).

The podophyllotoxin content of rhizomes of young plants (1-, 2- and 4-year-old) was found to increase with age in population growing at 2400 m (Purohit et al. 1999). The reported values for plants of any given age were higher than that found in the present study (Table 2.1). However, prior to this study, no information was available on the podophyllotoxin content in rhizomes, roots and leaves of plants of a known age series along with the growth parameters (including dry matter production) of seed-raised plants grown at a relatively lower altitude. Growing of alpine plants in accessible locations at near-natural conditions or at lower altitudes can be an overall strategy to promote their cultivation at relatively convenient locations, with a view to reduce pressure on the wild populations.

The podophyllotoxin content in rhizomes of another Himalayan species, i.e. *P. sikkimensis*, reported for the first time from our laboratory, ranged from 0.06 % to

0.73 % in plants collected from Thangu area of Sikkim in Eastern Himalaya (Paul et al. 2013). Though the levels are well below those reported in *P. hexandrum* or *P. peltatum*, estimations from other populations are required to be carried out in order to provide a clearer picture on its active ingredients.

2.3 Tissue Culture (In Vitro) Studies

2.3.1 *Aconitum balfourii*

Tubers (at the time of senescence of aerial parts) from an identified population of *A. balfourii* were collected from Kedarnath area (District Rudraprayag, Uttarakhand; 3300 m; 30°43′ to 30°45′ N and 79°3′ to 79°4′ E) and grown in the Institute Nursery at Kosi-Katarmal (District Almora, Uttarakhand; 1150 m; 29°38′15″ and 79°38′10′ E). Apical portions of fully grown plants were de-topped to encourage the growth of axillary buds. These buds (along with a small portion of the stem) were excised and used as explants which were disinfected and cultured as described earlier (Pandey et al. 2004). The explants were transplanted in test tubes containing 20 ml of Murashige and Skoog's medium (Murashige and Skoog 1962, MS) supplemented with sucrose (3 %, w/v), agar (0.8 %, w/v) and BAP (4.5, 13.5 or 22.5 μM); these explants were subcultured at least four to five times at 3 days interval to obtain contamination-free cultures. The axillary buds sprouted to form shoots and young leaves from these shoots were used for callus induction.

Callus formation occurred along the leaf margins within 5 weeks on the MS medium supplemented with various combinations of BAP (0.5–4.5 μM) and NAA (5.4–26.9 μM); maximum (75 %) explants with calli were obtained on MS medium supplemented with 4.5 μM BAP and 26.9 μM NAA. Hence, healthy and proliferating callus growing on this medium was transferred to the fresh medium containing different concentrations of BAP (4.5–22.2 μM) and NAA (0. 5–5.4 μM) for shoot induction. It was found that relatively lower levels of NAA (0.5–5.4 μM) and BAP (4.5 μM) resulted in excellent shoot regeneration. The adventitious shoot formation was maximum (100 %) on the medium supplemented with 4.5 μM BAP and 1.4 μM NAA, resulting in nearly six shoots/callus lump (Fig. 2.2a). Therefore, this medium was routinely used for shoot multiplication. Subsequently, single shoots were separated and subcultured on the MS medium containing BAP alone (0.5–44.4 μM) for shoot multiplication and elongation.

BAP at 1.1 μM concentration produced maximum number of shoots (24.7 per flask, 4.2 per cultured shoot/subculture) as well as resulted in shoot elongation (about 3.5 cm) within 4 weeks (Fig. 2.2b). The excised shoots developed on the MS medium supplemented with 1.1 μM BAP were placed on the medium supplemented with various concentrations of IBA for rooting. Root formation was initiated within 15–18 days in cultures supplemented with IBA (4.9, 12.3 or 24.5 μM), and maximum rooting (89 %) was observed on the medium containing 12.3 μM IBA (Fig. 2.2c). Well-rooted microshoots were first transferred to Erlenmeyer flasks containing sterilized soil rite, moistened with one-half MS salts and hardened for 2 weeks

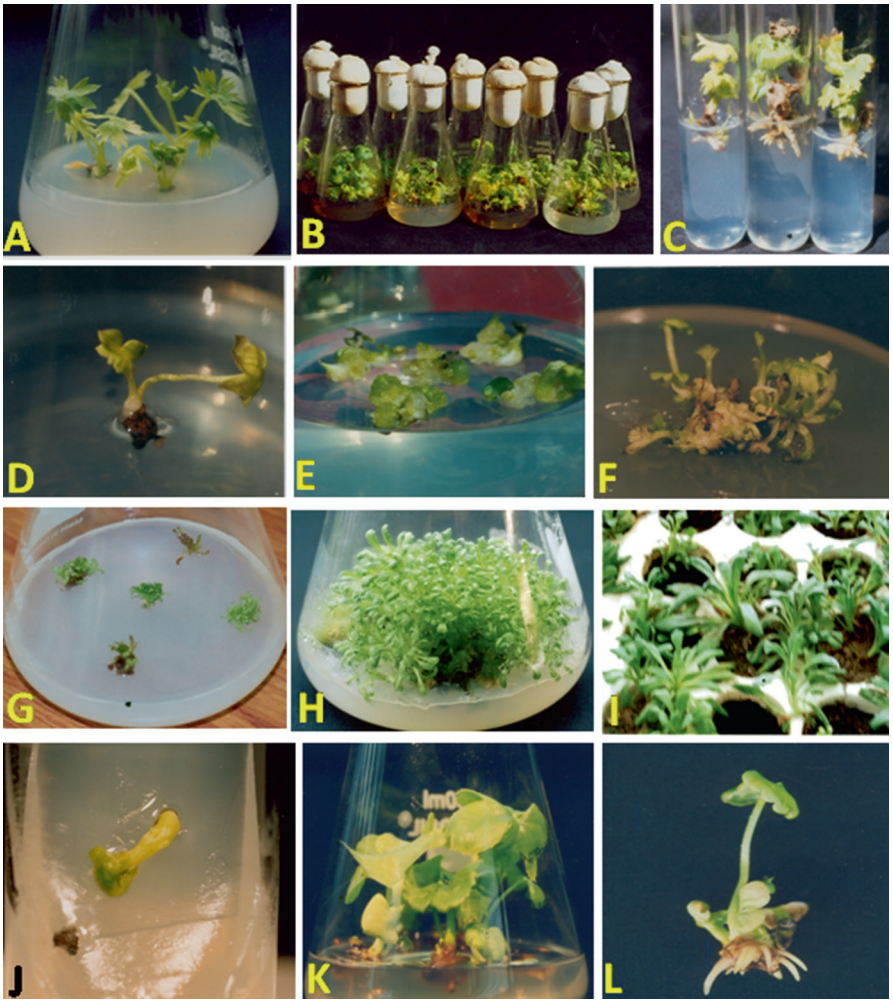

Fig. 2.2 Different stages during in vitro propagation of selected medicinal herbs: *Aconitum balfourii* (**a**–**c**), *A. heterophyllum* (**d**–**f**), *Picrorhiza kurrooa* (**g**–**i**) and *Podophyllum hexandrum* (**j**–**l**). (**a**) In vitro shoot formation on the MS medium supplemented with 4.5 μM BAP and 1.4 μM NAA; (**b**) shoot multiplication on the MS medium containing 1.1 μM BAP only; (**c**) rooting of shoots on the MS medium supplemented with 12.3 μM IBA after 4 weeks of culture; (**d**) germinated seed after 6 weeks of inoculation on the MS medium; (**e**) appearance of shoot budlike structures in cultures on the MS medium supplemented with 5.0 μM BAP and 1.0 μM NAA; (**f**) shoot multiplication from seedling explants (without radicle) on the MS medium containing 1.0 μM BAP; (**g**) initiation of shoot multiplication from cotyledonary node explants after 4 weeks of inoculation on the MS medium containing 1.0 μM BAP; (**h**) multiplication and profuse shoot formation on the MS medium containing 1.0 μM BAP; (**i**) hardened plantlets under greenhouse conditions, 4 months after ex vitro growth; (**j**) culture of excised embryos on the MS medium supplemented with BAP and IAA; (**k**) multiple shoot induction on the MS medium supplemented with BAP and IAA (1.0 μM each) and (**l**) induction of roots on shoots after transfer to the MS medium containing 0.5 μM IAA

under aseptic conditions in the culture room. These flasks were then shifted to the greenhouse (25 °C, RH 65 %) at Kosi-Katarmal and the plantlets transferred to plastic cups containing soil rite. Following 2 months of hardening (regular watering with one-half MS salts), the plants were transferred to a mixture of soil and FYM (1:1) and allowed to grow for another 2 months before transportation to their natural habitat. This substantially improved the ex vitro survival during hardening, and the roots were also found to proliferate (increase in number as well as in length) during this period of hardening. The survival of these plants was found to be 50 %, and 20 % of such plants formed normal tuberous roots within a year (Pandey et al. 2004).

Using the above-mentioned protocol, a callus lump (approx. size 9×8×5 mm, l×w×h) derived originally through a leaf explant (size 5×5 mm, l×w) of *A. balfourii* can produce up to six shoots directly through organogenesis, and each shoot can in turn provide four additional shoots per subculture, thus resulting in a total of 24 shoots within 9 weeks. Although an earlier study on this species (Singh et al. 1998) reported formation of embryos, adventitious shoots and roots, quantitative data on these aspects were not reported.

Quantitative analysis of diterpenoids by thin-layer chromatography followed by HPLC, in tubers of *A. balfourii* derived from seedlings and tissue culture-raised (TCR) plants showed that both aconitine and pseudaconitine (% of dry wt) were almost similar; aconitine content was 0.01 % in both types of plants, while pseudaconitine was 0.40 % in seedlings and 0.41 % in the TCR plants (Pandey et al. 2004). The results of the above study indicate that TCR plants compared favourably with the seed-raised plants of *A. balfourii*. More recently in vitro multiplication of this species was reported using both leaf and root explants taken from plant and passing through a callus phase; following shoot multiplication, root induction and hardening about 95 % of plantlet survival was observed in pots placed under greenhouse conditions (Bist et al. 2011; Sharma et al. 2012a). It would be important to add that the above-mentioned studies must be substantiated with DNA-based markers like RAPD, ISSR, etc. to confirm the genetic fidelity of these TCR plants.

In vitro studies in other species of *Aconitum* have also been carried out. As observed by Pandey et al. (2004), BAP has also been found to be effective in axillary bud proliferation and shoot multiplication in *A. noveboracense* and *A. napellus* (Cervelli 1987; Watad et al. 1995). Application of higher concentration of NAA with lower concentration of BAP has been reported to result in callus induction in *A. heterophyllum* (Giri et al. 1993). In *A. carmichaeli*, 22.1 μM BAP was extremely effective in stimulating the growth of shoot tip explants leading to formation of multiple shoots (Hatano et al. 1988), whereas a lower concentration (4.5 μM) of BAP produced greatest number of usable rosettes from the nodal explants in *A. napellus* and *A. noveboracense* (Cervelli 1987). Herbaceous species generally demand greater care, and therefore, during the initial phase of hardening, gradual removal of sucrose and salts helps the TCR plants to shift to autotrophic mode of nutrition (Bhojwani and Razdan 1996).

It should be mentioned that the electrophoretic pattern of storage proteins of TCR microtubers of potato was also found to be identical to that of field-produced tubers (Dodds et al. 1992). Similarly, it has been demonstrated that micropropagated

plants of *A. carmichaeli* showed less variation in alkaloids content when compared to those of field-grown plants (Hatano et al. 1988; Shiping et al. 1998).

The potential of using plant callus, cell and suspension cultures for long-term and sustainable production of active principles has been realized (Petersen and Alfermann 2001; Collin 2001, and references therein). Cell cultures have also been used extensively for biotransformation of various molecules to produce a variety of compounds of interest (Giri et al. 2001b). Recently, higher aconitine accumulation was reported in the presence of precursor acetyl-CoA (compared to control) when roots of *A. balfourii* were cultured in vitro (Sharma et al. 2014). Higher aconitine content (0.024 %) was also reported in roots of TCR plants when grown in hydroponics compared to TCR plants (0.012 %, Sharma and Gaur 2012). Besides, hairy root cultures induced by *Agrobacterium rhizogenes* have the ability of continued growth on plant growth substance-free medium and are known to produce elevated levels of active ingredients in several medicinal species (Giri et al. 1997, 2001a; Baiza et al. 1998). Studies on cell and hairy root culture in *A. balfourii* have not been reported so far.

2.3.2 *Aconitum heterophyllum*

Seeds of *A. heterophyllum* were collected from Kedarnath area (3300 m; District Rudraprayag, Uttarakhand) and stored at 4 °C (dark, 2 months). These were washed, surface disinfected, and inoculated (25 seeds/petri dish) onto the MS basal medium containing agar (0.8 %, w/v) and sucrose (2.0 %, w/v). Seeds were found to germinate within 5–6 weeks, and after another 3 weeks, the cotyledons were found to fully emerge (Fig. 2.2d). To initiate cultures, the cotyledons were separated from the seedlings and divided into two groups: the middle portion (where cotyledons unite in a sheathing base) and the outer portion (surrounding the middle region, Pandey 2002). Excised explants (middle/outer portions) were placed on the MS medium supplemented with NAA (5.0–25.0 μM) and BAP (5.0 μM) for callus induction. Pale-green calli were formed after 6 weeks of culture; in general, both types of cotyledon segments (middle and outer portions) exhibited similar performance. Maximum proliferation, i.e. 73 and 67 %, was observed in middle and outer portions, respectively, on the MS medium supplemented with 25.0 μM NAA and 5.0 μM BAP. Such callus maintained on this medium was later transferred to the medium containing 5.0 μM BAP and very low amounts of NAA (0.1–1.0 μM) for shoot initiation. The MS medium supplemented with 1.0 μM NAA and 5.0 μM BAP resulted in the formation of shoot budlike structures (Fig. 2.2e). These shoots did not last long and dried up within 3–4 weeks (Pandey 2002).

Germinated seedlings (radicle portion excised) containing cotyledons, hypocotyls, and hypocotyl sheath with apical meristem at the base were cultured on the MS medium containing BAP or thidiazuron (TDZ) for shoot proliferation. When the seedlings (without radicle) were cultured on the medium containing BAP (0.01, 0.1 and 1.0 μM), best response (80 %) was obtained on a medium containing 1.0 μM BAP with maximum shoot formation (six shoots/explant) after 8 weeks (Fig. 2.2f).

These shoots were excised from the clumps, and individual shoots were then cultured on the medium containing 1.0 μM BAP for multiplication. On the other hand when the seedlings (without radicle) were cultured on the medium containing TDZ (0.1, 1.0, and 10.0 μM), it resulted in early emergence of leaves. The use of 0.1 μM TDZ resulted in the emergence of the first leaf after 9 days of incubation in comparison to 32 days in control; the second leaf was found to emerge within 16 days in comparison to 42 days in control. However, the shoots thus formed could not survive for long and died within 2–3 weeks (Pandey 2002).

Although attempts were made in this investigation to obtain microshoots for further growth and multiplication, success could not be achieved. Further attempts are underway to obtain improved growth of such microshoots. The cytokinin, BAP, has been found to be quite useful for shoot bud proliferation and shoot multiplication in several species of *Aconitum*, including *A. heterophyllum* and *A. balfourii* (as mentioned above). It is worth mentioning that in vitro propagation of *A. heterophyllum* has been reported (Giri et al. 1993; Jabeen et al. 2006), and these workers have also used BAP to obtain shoot formation; mass scale propagation of plants has, however, not been reported.

The establishment of hairy root cultures in *A. heterophyllum* and the production of active ingredients were successfully demonstrated (Giri et al. 1997). They reported that the total aconitine content of transformed roots was 3.75-fold higher than that of non-transformed roots. However, further reports on the production of active compounds either by hairy roots or through cell culture have not appeared. Recently, the protocol for in vitro plant regeneration in *A. violaceum*, another Himalayan medicinal herb, has been demonstrated (Mishra-Rawat et al. 2013 a, b).

2.3.3 *Picrorhiza kurrooa*

Seeds of *P. kurrooa* were collected from Pindari area (3400 m; District Bageshwar, Uttarakhand; 30°6′ to 39°15′ N and 70°55′ to 80°5′ E); these were removed from the spikes, air dried, and stored at 4 °C (dark, 3 months). Seeds were then washed, surface disinfected and inoculated (25 seeds/petri dish) on the MS basal medium containing agar (0.8 %, w/v) as described earlier (Chandra et al. 2004, 2006). Germinated seeds were used for obtaining ‘cotyledonary node’ or ‘shoot tip’ explants.

Two weeks following seed germination under in vitro conditions, the radicle, cotyledons and a part of the hypocotyl (3.0–5.0 mm below the cotyledonary node) were removed from the seedlings, and the remaining portions of the explants containing a small part of the hypocotyl and the cotyledonary node (cotyledonary node explant) were cultured on the MS medium supplemented with BAP or kinetin (Kn, 1.0–10.0 μM). The other set of germinated seeds were allowed to germinate in vitro and grown (avg. height of seedlings 2.5 cm) till the first leaf emerged. ‘Shoot tip’ portions (0.5 cm) were carefully excised from seedlings, under aseptic conditions, and further subcultured on the MS medium supplemented with BAP or Kn (0.1–10 μM).

The response of cotyledonary node on the medium supplemented with various levels of cytokinins (BAP or Kn) resulted in multiple shoot formation within 3–4 weeks on the medium containing 1.0 μM BAP or 2.5 μM Kn (Fig. 2.2g). The maximum (66.7 %) proportion of explants were found to form healthy shoots (4.8 shoots per explant) on the MS medium containing 1.0 μM BAP, while the maximum number of shoots (5.5 per explant) was observed on the medium with 1.0 μM Kn. Subculturing was carried out at 4–6 weeks interval on the medium containing 1.0 μM BAP in order to obtain good and steady supply of shoots, devoid of any callus formation at the base of the shoots.

Shoot tip explants also proliferated and formed multiple shoots (within 4 weeks) on the MS medium irrespective of the concentration of BAP or Kn (up to 2.5 μM) used; higher concentrations (5.0 and 10.0 μM) of these cytokinins were, however, ineffective. All the explants (100 %) cultured on the medium supplemented with 1.0 μM BAP developed multiple shoots, while 53.3 % explants were found to form multiple shoots using the same concentrations of Kn. The maximum number of shoots (ca. 12 per explant) was obtained on the medium containing 1.0 μM BAP (Fig. 2.2h). The average length of shoots was also higher (4.0 cm) on this medium. The minimum shoot formation (average <1.0) was found when 0.1 μM Kn was used (Chandra et al. 2004).

Transfer of individual shoots (3.0–5.0 cm height) to PGR-free medium resulted in over 60 % rooting (without callus formation) after 11 days of inoculation. However, the rooting efficiency could be improved (up to 100 %) by addition of any of the three auxins, namely, NAA, IBA or IAA (0.1, 0.5, 2.5 μM) added to the MS medium. The mean number of roots formed per shoot was highest (8.3) when 0.5 μM NAA was used. The rooted plants were transferred to thermocole trays or cups containing a mixture of soil and sand (2:1, v/v) and placed for hardening in a greenhouse (25 °C; RH, 90 %) at Kosi-Katarmal (1150 m). The plantlets (Fig. 2.2i) were found to grow normally after 4 months, under greenhouse conditions; these were then transferred to high-altitude experimental site in village Khaljhuni (2450 m), hardened for a week in a polyhouse and field transplanted in plots for assessing growth and performance (Chandra et al. 2004).

In this study multiplication of *P. kurrooa* has been achieved using both cotyledonary nodes and shoot tips as explants taken from in vitro-raised seedlings. Cotyledonary node explants from in vitro-grown seedlings, like in the present study, have exhibited organogenic competence in several herbaceous species (Mallick and Rashid 1989; Jackson and Hobbs 1990). Cytokinins like BAP or Kn, at lower concentrations, proved to be extremely effective for induction of multiple shoots and subsequent shoot multiplication in both the types of explants used. In previous reports on in vitro propagation of *P. kurrooa*, these two cytokinins were also found to induce shoot multiplication in explants taken from a mature plant (Lal et al. 1988; Upadhyay et al. 1989). In the present study, shoot tips were found to be superior for obtaining multiple shoots; the maximum number of shoots was found on the medium containing BAP. Moreover, the higher concentration of BAP resulted in hyperhydric (vitrified) shoots during subsequent subcultures. Lowering the cytokinin concentration in the medium resulted in regenerating normal shoots from the

base of vitrified shoots. Vitrification of the shoot in cytokinin-supplemented medium has also been reported earlier in *P. kurrooa* (Upadhyay et al. 1989).

Rooting of microshoots has been reported in *P. kurrooa* by incorporation of various auxins, namely, NAA, IBA and IAA into the rooting medium (Lal et al. 1988; Upadhyay et al. 1989). While the report by Lal et al. (1988) did not indicate rooting percentage, 89 % rooting was achieved in a subsequent study using the MS medium supplemented with 1.0 μM NAA, with root initiation taking place after 20 days (Upadhyay et al. 1989). In the present investigation, auxins were found to induce cent per cent rooting in microshoots. Furthermore, the time required for root initiation was also reduced (8 days) when a lower concentration (0.1 μM) of NAA or IBA was used; this also resulted in minimizing or avoiding callus formation at the base of the shoots.

It is important to mention that following in vitro multiplication, microshoots of *P. kurrooa* could also be encapsulated; assessment of genetic fidelity of proliferating microshoots indicated true-to-type nature and compared well with the mother plant (Mishra et al. 2011a). This technology will be useful for storage and transport of microshoots for plantation at a later period when the climatic conditions become congenial at higher altitudes. In another study, Sood and Chauhan (2009) reported high-frequency callus induction and subsequent plant regeneration from various explants of *P. kurrooa*.

A rapid and efficient tissue culture protocol was developed (Bantawa et al. 2010) for an elite clone of another important Himalayan species of *Picrorhiza*, i.e. *P. scrophulariiflora* using explants taken from rhizome explants on woody plant medium supplemented with BAP and Kn. About 90 % of the regenerated microshoots could be rooted using NAA, without basal callus formation. Subsequently more than 1000 plants were hardened and field planted under natural conditions (Bantawa et al. 2010).

In addition to the above studies, it has been successfully demonstrated that hairy root lines of *P. kurrooa* produced elevated levels of active ingredients (Verma et al. 2007; Anonymous 2010; Mishra et al. 2011b). Such studies would offer possibilities for in vitro commercial production of important metabolites and assist in the conservation of important and/or endangered Himalayan medicinal plants.

2.3.4 *Podophyllum hexandrum*

Seeds of *P. hexandrum* Royle were collected from the Pindari region (3260 m; District Bageshwar, Uttarakhand) and stored at 4 °C (2 months). These were then washed with water, surface disinfected and allowed to imbibe in sterile distilled water (overnight) as described earlier (Nadeem et al. 2000). The embryos were carefully excised and transferred onto the MS medium containing 3.0 % (w/v) sucrose and 0.8 % (w/v) agar and supplemented with various concentrations of PGSs [BAP (0.5–5.0 μM), IAA (1.0–4.0 μM) or NAA (0.5–5.0 μM)] and activated charcoal (0.4–1.0 %, w/v). Excised embryos germinated within 7 days of inoculation on the basal medium or on the medium supplemented with BAP (0.5–4.0 μM);

a prominent cotyledonary tube with cotyledonary leaves and a distinct radicular portion were observed (Fig. 2.2j).

When the excised embryos were placed on the medium supplemented with 1.0–4.0 μM IAA and 1.0 μM BAP, multiple shoots were formed. The highest shoot multiplication rate (5.0 shoots/embryo) was observed on the medium containing both IAA and BAP (1.0 μM each, Fig. 2.2k). The base of the cotyledonary leaf in the embryos swelled to give rise to multiple shoots in about 4–5 weeks. These shoots were then separated and cultured individually for root induction. While the basal medium did not induce rooting, reducing its strength by half resulted in rooting of 16.6 % shoots. However, rooting increased only slightly (25 %) when the medium was supplemented with 0.5 μM IAA (Fig. 2.2l, Nadeem et al. 2000).

The well-rooted microshoots were transferred into the 250 ml flasks containing sterilized vermiculite, moistened with one-half MS salts, and allowed to harden for 15 days under aseptic conditions in the culture room. The plantlets were then transferred to polybags containing vermiculite and kept in a polyhouse (25–30 °C, 50 % shading with green/black net) for 30–35 days; the polybags were covered with polythene from the top to maintain high humidity (>90 % RH). Subsequently the plants were transferred to polybags containing soil and kept in the same polyhouse for another 3 months; during this period all the hardened plants showed new shoot emergence and behaved like normal field-grown plants (Nadeem et al. 2000).

The radicular, cotyledonary tube and cotyledonary leaves of germinated embryos were dissected and also cultured separately on the MS medium containing varying combinations and concentrations of NAA, BAP and GA_3. Callusing was induced from the basal end of embryos in most combinations. The calli so obtained remained greenish to creamish white up to the time of the first subculture; some calli, however, turned yellow and friable following the second subculture. Subsequently, somatic embryogenesis was recorded when the callus was subcultured on the medium supplemented with 5.0 μM NAA and 0.5 μM BAP. For maturation of somatic embryos, calli bearing different stages of embryos were transferred to various media combinations (MS, one-half MS, MS + charcoal and MS + 0.5 μM NAA). Irrespective of various combinations tried, nearly all somatic embryos were found to mature. Although somatic embryos of different sizes were obtained, the smaller ones turned green after a week on the MS medium supplemented with 0.5 μM NAA. The radicular portions elongated on the medium containing activated charcoal in 2–3 weeks, and these could be made into ‘synthetic seeds’ by encapsulation in sodium alginate beads. Subsequently, these could be germinated to form complete plants on a medium containing 1.0 μM BAP and 2.5 μM GA_3 (Nadeem et al. 2000). The plants were then hardened as mentioned above.

Tissue culture studies on *P. hexandrum* have been reported earlier (Arumugam and Bhojwani 1990); while multiple shoot formation from zygotic embryos was demonstrated, rooting of these shoots was not reported by these authors. The study by Nadeem et al. (2000) outlined above seems to be the first report of in vitro propagation of this species via multiple shoot formation and subsequent rooting. Addition of 0.5 μM IAA to the MS medium enhanced the rooting process. Somatic embryogenesis followed by subsequent germination is also beneficial for propagation,

because it eliminates the additional step of root induction that is required in propagation through multiple shoot formation (also see Nadeem et al. 2000). The TCR plants have been successfully grown and maintained in pots; these plants exhibited winter dormancy behaviour similar to that of field-raised plants and remained healthy. In a later study also using rhizome explants, in vitro plantlet regeneration in *P. hexandrum* was achieved via direct organogenesis (Chakraborty et al. 2010). These workers used the MS medium supplemented with NAA and BAP for shoot regeneration, and after multiple shoot formation in a medium containing IAA, the microshoots were rooted on one-half MS liquid medium containing IBA (Chakraborty et al. 2010). However, subsequent field transfer of these TCR plantlets was not reported. In spite of the efforts on the development of TC protocols for *P. hexandrum* (above-mentioned studies) and *P. peltatum* (Sadowska et al. 1997; Moraes-Cerdeira et al. 1998; Kim et al. 2007), large-scale propagation and cultivation trials of such plants have not been reported. Efforts must, therefore, be made to undertake large-scale multiplication of elite clones as well as conduct cultivation trials of these two species.

Production of podophyllotoxin by callus, root, suspension and hairy root cultures has also been demonstrated. Kadkade (1981, 1982) reported podophyllotoxin production (up to 0.65 %) from callus cultures of *P. peltatum*. Moreover, cell lines of the same species with the capacity to produce podophyllotoxin were also reported (Kutney et al. 1991). Subsequently embryogenic cell and adventitious root culture systems in *P. peltatum* were reported to produce podophyllotoxin, the later producing higher levels (Anbazhagan et al. 2008). Callus and suspension cultures derived from *P. hexandrum* were shown to produce podophyllotoxin (Van Uden et al. 1989, 1990; Woerdenberg et al. 1990; Chattopadhyay et al. 2001, 2002). Later Li et al. (2009) demonstrated an improved and effective method of podophyllotoxin production by root cultures of *P. hexandrum*. Hairy roots of *P. hexandrum* were also demonstrated to produce podophyllotoxin (Giri and Narsu 2000; Giri et al. 2001a); however, the levels reported were low. This alternate and continuous method has the potential for commercial production of high-value podophyllotoxin.

Another alternative approach for the production of podophyllotoxin through endophytic fungi is being vigorously perused nowadays. Ever since reports on taxol production by endophytic fungi isolated from yew species (Stierle et al. 1993; Strobel et al. 1996), there has been a growing interest in the biosynthetic capabilities of endophytes which are considered to be rich and diverse source of natural products. An endophyte isolated from *Nothapodytes foetida* was reported to produce camptothecin (Puri et al. 2005). Later the same group isolated an endophytic fungus *Trametes hirsuta* from *P. hexandrum*, as a novel alternative source of podophyllotoxin and related aryl tetralin lignans (Puri et al. 2006). Further, two strains (PPE5 and PPE7) of the fungus *Phialocephala fortinii* Wang and Wilox capable of podophyllotoxin production were isolated from *P. peltatum* (Eyberger et al. 2006). More recently, isolation of an endophytic fungus, *Fusarium solani*, from *P. hexandrum* collected from Kumaun region of Indian Himalaya, and podophyllotoxin production were reported (Nadeem et al. 2012). It is interesting to mention that isolation and identification of an endophytic strain of *Fusarium oxysporum* producing

podophyllotoxin were reported from *Juniperus recurva* (Kour et al. 2008). In view of the low amount of podophyllotoxin production by endophytes, their role needs further investigation. Moreover, optimization of conditions and isolation of high-yielding strains are essentially necessary to improve production.

2.4 Field Plantations and Performance

Field plantation and further assessment of plant performance (raised by tissue culture as well as conventional methods) were carried out at a high-altitude experimental site in village Khaljhuni (District Bageshwar, Uttarakhand; 2450 m; 30°6′12″N, 79°58′29″ E; Fig. 2.3a–c).

2.4.1 Soil and Climate Condition

Soil in the plantation site was found to be acidic (pH 5.7) with average soil moisture content of 23 % (April to June). Nitrogen, phosphorus and carbon contents were recorded as 0.39, 0.006 and 3.0 % (dry weight basis), respectively. The mean maximum temperature during winter months (October 2003 to March 2004) was 14.3 ± 2.8 °C and the minimum was 7.2 ± 1.2 °C, whereas during summer months (April 2003 to September 2003), these were 25.9 ± 0.4 °C and 16.0 ± 1.5 °C, respectively.

2.4.2 Field Preparation and Plantation

Field plantation to assess growth and biomass production was conducted in the cultivated land of a local farmer at village Khaljhuni (2450 m). Keeping in mind the land topography and small size of plots, separate plots were used for each species. The land was ploughed, levelled and raised and plain beds were prepared (Pandey 2002; Chandra 2002).

2.4.3 Plantation in the Field

2.4.3.1 *Aconitum balfourii*

Plants [TCR and seed raised (SR); grown in Kosi-Katarmal, Almora, transported and hardened at Khaljhuni] were planted in raised beds of 5 × 4 m (length x width). The spacing between rows as well as between plants was 15 cm. The growth of TCR plants was slow with 50 % survival in the first 3–4 months; subsequently the remaining plants also failed to survive. The growth and survival rate of seed-raised (SR) plants was 80 % till the end of the growing season (Fig. 2.3e, Pandey 2002).

In addition, tubers from field-grown plants were cut transversally into three segments of equal length, i.e. top, middle and basal, and planted in field plots of

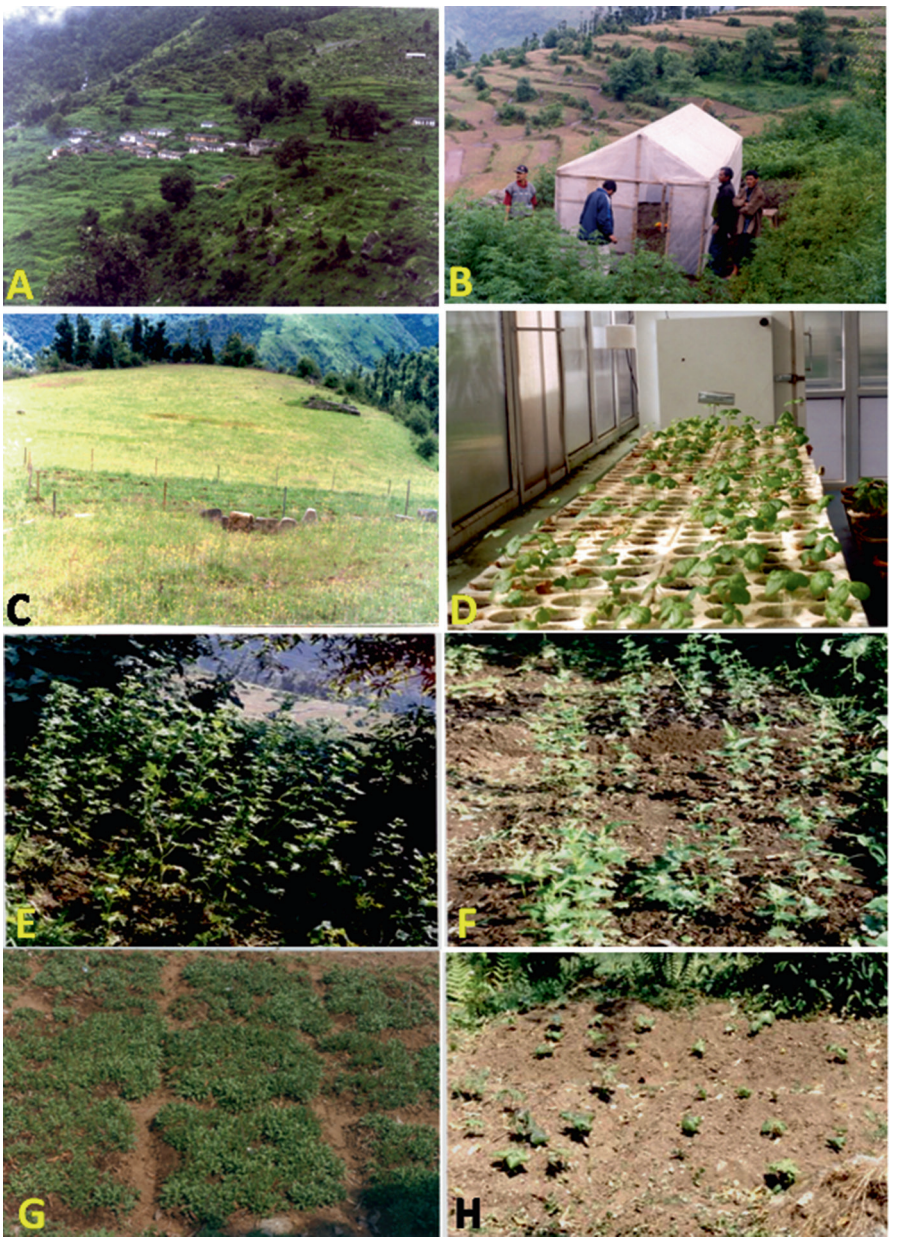

Fig. 2.3 Field plantation of selected medicinal herbs in a high-altitude field station located at Khaljhuni (2450 m). (**a**) A view of village Khaljhuni; (**b**) view of a polyhouse in the nearby area used for raising and hardening of plants; (**c**) fenced area of demonstration plots; (**d**) seedlings of *P. hexandrum* raised in the greenhouse at Kosi-Katarmal (1150 m); (**e**–**h**) demonstration plots of different herbs; (**e**) *Aconitum balfourii* (seed-raised plants); (**f**) *A. heterophyllum* (seed-raised plants); (**g**) *Picrorhiza kurrooa* (in vitro-raised plants) and (**h**) *Podophyllum hexandrum* (seed-raised plants)

15 × 7 m (length × width). The spacing between rows was 30 cm whereas between plants was 15 cm. The plants exhibited satisfactory growth during the season; however, tuber formation was hampered at the end of the season and the tubers could not be located during the next year (April, Pandey 2002).

2.4.3.2 *Aconitum heterophyllum*

Since TCR plants of *A. heterophyllum* were not available, SR plants (raised at Kosi-Katarmal and hardened at Khaljhuni as in *A. balfourii*) and tuber segments (taken from natural population) were planted in plots of 5 × 4 m (length × width). Tubers were cut into uniform-sized segments and planted with spacing as in the case of *A. balfourii* seedlings and tubers. Like *A. balfourii*, the plants exhibited 80 % survival during the first 3–4 months followed by satisfactory growth till the end of the growing season (Fig. 2.3f); however, tuber formation was hampered at the end of the season and the tubers could not be located during the next year (April, Pandey 2002).

2.4.3.3 *Picrorhiza kurrooa*

Plants (TCR and SR) of *P. kurrooa* (raised in Kosi-Katarmal and then hardened in Khaljhuni) were planted in plain and raised beds of 6 m length and 4 m width. The spacing between rows as well as between plants was 15 cm. Nine-month-old (4 months in the greenhouse and 5 months in the experimental field) SR and TCR plants were compared for growth in terms of plant height, number of leaves, runner biomass, etc. (Chandra 2002). It was observed that the average plant height was higher (7.1 cm) in TCR plants (Fig. 2.3g) in comparison to SR plants (5.5 cm), but the runner diameter was higher in SR plants (2.0 mm as compared to 1.7 mm in TCR plants). The total biomass (dry weight of runners) per plant was also higher for SR plants (3.5 g as compared to 2.4 g in TCR plants). The leaf parameters were also recorded, and thicker leaves were found in TCR plants. Higher leaf area and specific leaf mass were recorded in SR plants (3.7 cm^2 and 3.5 mg/cm^2, respectively, as compared to 2.2 cm^2 and 2.9 mg/cm^2 for TCR plants). Among other parameters studied, chlorophyll 'a' content was found to be higher for seedlings (0.82 mg/g FW as compared to 0.69 mg/g FW in TCR plants), while chlorophyll 'b' content was slightly higher in TCR plants. Per cent relative water content (RWC) of leaves was essentially similar for both sets of plants (Chandra et al. 2004).

In addition, runner segments obtained vegetatively from the field-grown plants (containing three nodes, 5.0–6.0 cm long) were planted in plain and raised beds of 30 m length and 16 m width. Similarly, seedlings that were raised in trays or in beds under polyhouse conditions were transplanted to field beds containing farm yard manure during the month of March in plain and raised beds. The spacing during planting was kept as mentioned above. Runner cuttings were more successful for multiplication as well as for higher biomass production within a short period than cultivation through seeds. Both types of plants showed satisfactory growth and multiplication in the field. In the following year (April), new shoots developed from these runners and plants were found to grow well during the second year (Chandra 2002).

2.4.3.4 *Podophyllum hexandrum*

Plants (TCR and SR) grown in Kosi-Katarmal (Fig. 2.3d) and then hardened at high altitude (Khaljhuni) were planted in plots of 7 × 4 m (length × width). The spacing between rows as well as between plants was 15 cm. It was observed that the growth of TCR plants was slow with only 30 % survival up to the first 3 months; subsequently there was no survival. The survival rate of SR plants (Fig. 2.3h) was also very low (20 %) till the end of the growing season, and rhizome formation was not observed in the next growing season (Pandey 2002).

Rhizome segments of (4.0–5.0 cm long transverse segments containing axillary buds in each segment) were planted in nursery beds of 5 m length and 8 m width. The spacing between rows was 20 cm, whereas the spacing between plants was 15 cm. One-year-old seed-raised plants were also planted in plots of 4 m length and 2 m width with similar spacing as for the rhizome segments. The growth and survival of these segments/plants was unsatisfactory and rhizome formation was not observed during the next growing season (Pandey 2002).

2.4.4 Weeding, Irrigation and Fungicide Treatment

Manual weeding was carried out at regular intervals, following weed growth. Irrigation, particularly in peak summer months (May), was done as and when required according to the species and the status of soil moisture content (Chandra 2002). In general, no infection occurred, but in plots of *P. kurrooa*, infection with powdery mildew was observed at the time of pre-senescence (July–August). These plots were treated with a systemic fungicide, Bavistin (1 %, w/v), by manual spraying at 48 h interval (thrice).

2.4.5 Harvesting

After completion of the reproductive phase, *P. kurrooa* plants were found to be mature for harvesting (during September). The TCR as well as the SR plants did not flower in the first season. Thinning of runners was done in the field itself; the roots along with runners were dug and excised with a sharp knife. The excised runners were planted in separate plots to get another crop (Chandra 2002).

Efforts were made to grow TCR plants along with SR plants of the target species on the farmer's field located at a higher altitude and assess their growth and performance. This is possibly the first report wherein attempt has been made to assess the field performance of TCR plants. Although TCR plants were subjected to sufficient hardening in the greenhouse and then further acclimatized at the high-altitude field station prior to plantation, among the four species tried, performance of only *P. kurrooa* was satisfactory; survival of the other three species was poor. It must be mentioned that during hardening under the greenhouse, TCR plants of *P. kurrooa* were inoculated with some bacterial isolates (e.g. *Bacillus subtilis*) having antagonistic properties against pathogenic fungi (Chandra et al. 2004). This step is important as

maximum mortality is generally caused by fungal infection during early weeks of transfer of rooted plants to the soil. The survival rate of plants (both TCR as well as SR) in the field can perhaps be improved if the time taken during transport of plants from the greenhouse to the field planting is reduced. Since the field is located in a distant and difficult-to-reach place in the subalpine Himalayan zone, a major climatic shift, in addition to transport shock, may have affected the final survival of plants in the field.

It needs to be mentioned that due to shortage of TCR plants, further field trials could not be carried out in the next season (second year). While in vitro (tissue culture) technology has potential for large-scale production for subsequent commercial cultivation, and based on the success recorded with *P. kurrooa*, concerted efforts are required for the remaining three species. The four target species are listed under 'vulnerable', 'endangered' or 'critically endangered' category and their export is regulated. All these four species are currently in the 'List of medicinal plants prohibited' of the Ministry of Commerce, Government of India (Lakhanpal 1998). In spite of this, only limited attempts have been made to develop agrotechnologies to promote their cultivation. Agrotechnologies for some medicinal herbs, including *A. heterophyllum*, *A. balfourii* and *P. kurrooa*, have been reported using seed, tubers and runners as propagules (Purohit 1997; Maikhuri et al. 1998). Thus, tissue culture propagation protocols reported in this communication, along with the conventional methods, would be useful in initiating programmes on systematic cultivation with proper coordination and linkages between the government, state forest departments, State Biodiversity Boards, state medicinal plant boards, researchers and farmers; the efforts should be directed towards ensuring long-term benefits to the local community, as well as providing protection to wild populations of such important plants.

2.5 Conclusions

Medicinal and aromatic plants form an integral and essential part of the lives of hill communities, and the inhabitants depend on these plants for use in healthcare. These plants are well-known source of active principles in Ayurvedic, Unani and other traditional systems of medicines. At present these plants are major sources of many high-value drugs. Concomitant with the ever-increasing global demand for the 'naturals', these species are being subjected to reckless, often illegal harvesting, well beyond their natural regeneration capacity. The medicinal/pharmaceutical properties are mainly attributed to secondary metabolites. Various investigations have indicated a wide variation in active ingredient content in plant parts among the populations of the same species, and hence exact knowledge of its content is extremely important. Our findings clearly support this for all the species described above. Besides the known source of ingredients, i.e. underground parts, other plant parts (i.e. stem and leaf) and alternative means (callus, cell and suspension culture and hairy roots) of obtaining these compounds must be exploited. The above investigations suggest that besides rhizomes/roots, leaves of *P. hexandrum* can be harvested and thus the plants can be a renewable source of podophyllotoxin. It is

well known that in vitro (tissue culture) techniques are an alternate and effective means of propagation. These are being used not only in forestry, horticulture, land rehabilitation and other afforestation programmes but can facilitate the recovery of rare and endangered plant species (Bajaj 1989; Palni et al. 1998; Nadeem et al. 2000; Nandi et al. 2002; Pandey et al. 2002; Chandra et al. 2006; Purohit et al. 2015). However, it must be emphasized that tissue culture-raised plants need to be carefully hardened and acclimatized. The overall success would depend upon efficient field transfer, following proper hardening (using both physical and biological means), continued monitoring of growth, regeneration ability of rhizomes and periodic estimation of active ingredients. Since in vitro techniques are known to induce variability, the plants so raised should be screened for useful somaclonal variants, which may be exploited for obtaining plants or cultures with high secondary metabolite content. Moreover, this technique is used for rapid supply of large number of desired quality planting material (clone) which is phenotypically uniform and genetically akin to the mother plants. Thus determination of genetic fidelity of TCR plants is extremely important for validating true-to-type or variant nature of clones. Molecular markers have been routinely applied to detect genetic integrity among TCR plants and have been successfully carried out in tea (Devarumath et al. 2002; Mondal and Chand 2002; Bag et al. 2008), bamboo (Das and Pal 2005; Agnihotri et al. 2009), medicinal plants of IHR (Mishra et al. 2011a; Giri et al. 2011; Purohit et al. 2015) and several other species.

As most alpine medicinal plants require several years to reach optimal harvestable size, in vitro techniques can be effectively applied for rapid and clonal propagation and thus pave the way for conservation along with economic exploitation. Data on morphological and some physiological parameters of field-planted TCR *P. kurrooa* plants showed comparable results like those of seedlings/mother plants (this study). Similar results have also been reported for a temperate bamboo, *Thamnocalamus spathiflorus* (Bag et al. 2000) and 'maggar' bamboo, *Dendrocalamus hamiltonii* (Agnihotri et al. 2008, 2009; Bag et al. 2012). Moreover, reports of occurrence of podophyllotoxin in leaves and stems of *P. hexandrum* from various populations of Indian Central Himalaya in our laboratory (Pandey 2002; Pandey et al. 2013) are significant as leaves can be a renewable source of podophyllotoxin and can be exploited instead of harvesting the entire plant. The outcome of the above-mentioned studies is likely to result in the development of technology packages for mass scale propagation and cultivation of these species following proper field trials. This will not only help in restoration of degraded lands but also result in deriving economic benefits by the local communities.

Acknowledgements The financial support was received from the Department of Biotechnology, Govt. of India, New Delhi, in the form of projects (BT/PR/502/Agr/08/39/96 and BT/PR/3447/AGR/16/285/2002-VI), and fellowships to HP and BC are duly acknowledged. Director, GBPIHED is thanked for support and encouragement. The Ministry of Environment, Forest and Climate Change, Govt. of India is thanked for providing facilities and Drs Anil Kumar and R.K. Agnihotri for helping in HPLC analyses and preparing the photoplates, respectively.

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http://www.springer.com/978-981-10-1916-6

Plant Tissue Culture: Propagation, Conservation and Crop Improvement

Anis, M.; Ahmad, N. (Eds.)

2016, XVII, 621 p. 79 illus., 66 illus. in color., Hardcover
ISBN: 978-981-10-1916-6