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REVIEW

Picrorhiza kurrooa: current status and tissue culture mediated biotechnological interventions

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Abstract *Picrorhiza kurrooa*, one of the important plant species among the various medicinal plants, is endemic to Himalaya. As the plant is useful in the treatment of various diseases, e.g., hepatic disorders, gastric troubles, anemia, asthma, etc., illegal collection from the wild is increasing and now this plant is banned for export in any form and listed as 'endangered'. Ecological studies carried out on this species in last few decades suggested that the availability of this species in its specific habitats is comparatively lower than other associate species. Possible factors responsible for this depletion are increasing demand in the pharmaceutical industries, habitat specificity, heavy exploitation from the wild, unorganized cultivation practices etc. Biotechnology is playing a crucial role to conserve this important plant species. The past 23 years have witnessed a progressive biotechnological advances made in *P. kurrooa*. People have published various reports on establishments of in vitro culture techniques including micropropagation, synthetic seed production, plant regeneration via callus-mediated shoot organogenesis, adventitious shoot regeneration, genetic transformation through *Agrobacterium rhizogenes*, secondary metabolite analysis etc. This review attempts to focus on present ecological status and provide a comprehensive account on the tissue culture-mediated biotechnological interventions made in *P. kurrooa* for improvement and conservation of this medicinally important plant.

Keywords *Picrorhiza kurrooa* · *Agrobacterium rhizogenes* · Conservation · Endangered · Secondary metabolite

Abbreviations

MS	Murashige and Skoog medium
BAP	6-Benzylaminopurine
IAA	Indole-3-acetic acid
NAA	α-Naphthaleneacetic acid
2,4-D	2,4-Dichlorophenoxyacetic acid
Kn	Kinetin
IBA	Indole-3-butyric acid
GA_3	Gibberellic acid
m amsl	Meter above mean sea level
PCR	Polymerase chain reaction
RAPD	Random amplified polymorphic DNA
ISSR	Inter simple sequence repeat
UPGMA	Unweighted pair-group method with arithmetic mean

Introduction

The Himalaya has always been a source of important medicinal plants for local medicinal practitioners,

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vaidyas and plant explorers since time immemorial (Samant et al. 1998). During the past two decades, all the human activities which lead to the destruction of plants from the wild and demands from the pharmaceutical industry for domestic needs as well as for exports have resulted in scarcity of medicinal plants species in the wild (Kala et al. 1998; Dhar et al. 2000). Declining population of threatened medicinal plants in the wild, illegal collection and overexploitation is hot issue among various concerned departments and workers (Jacobson et al. 1991; Sheldon et al. 1998; Dhar et al. 2000). Among many factors involved in the low number of species in the wild, restricted distribution and anthropogenic disturbances like over exploitation, harvesting, trampling, small population size etc. are most important (Semwal et al. 2007). Generally, the medicinal plant collectors are unaware about the proper collection and storage practices (Maikhuri et al. 1998; Nautiyal et al. 2001), which results in destructive harvesting and finally leads to the poor natural regeneration. This consequently leaves very little scope for the natural regeneration of the species (Rao et al. 2000). Furthermore, the removal of the entire plant before seed maturation reduces the possibility of seed development for future regeneration (Sheldon et al. 1997). In many cases, most of the species are regarded as the only source of important drugs, so they are heavily exploited at commercial scale. Thus, the problem not only results in the removal of plant material but also causes lack of quality plant material. As the sources of elite plant material are getting depleted, there is a need for rapid multiplication and conservation of important medicinal plants. However, in last few years, emphasis was laid on sustainable cultivation via new agro-techniques to reduce the pressure on important medicinal plants, but no encouraging results were achieved.

Besides, the conventional breeding and propagation techniques have contributed significantly to the growth of pharmaceutical industry over the past several decades in a variety of ways including the varietal improvement. However, in view of the limitations of conventional breeding methods coupled with the demand for vertical increase in productivity at affordable cost of production, application of biotechnology-based tools provides attractive alternative approaches. In this regard, plant propagation via tissue culture has been evolved as a valuable method compared to conventional plant multiplication in last few decades.

Number of studies based on micropropagation, synthetic seed production, callus-mediated shoot organogenesis, adventitious shoot regeneration, genetic transformation etc. of *P. kurrooa* have been published (Lal et al. 1988; Upadhyay et al. 1989; Chandra et al. 2006; Verma et al. 2007; Sood and Chauhan 2009a, b; Mishra et al. 2011a, b); however, keeping in mind the increasing demand in the market and consequently increasing threats on natural habitats, importance and need of biotechnological intervention have not been reviewed yet. The extent of research in terms of explored and remaining ecological and biotechnological works in *P. kurrooa* is depicted in Fig. 1. The present review attempts to provide a detailed account of ecological status and tissue culture-mediated biotechnological interventions in *P. kurrooa* to highlight the areas of tissue culture explored so far and to emphasize over the areas not attempted yet. This review also indicates if unexplored areas like metabolic engineering are taken into consideration, might change the face of future research focused on *P. kurrooa*.

Picrorhiza kurrooa at a glance

Picrorhiza is a small genera belonging to the tribe Veroniceae of the family Scrophulariaceae. This family is, according to the taxonomical system of Cronquist, arranged in the order scrophulariales, subclass Asteridae, class Dicotyledonae, of the Angiospermae. The genus *Picrorhiza* was considered monotypic, with as only species *P. kurrooa*, until Pennell (1943) distinguished a second species: *P. scrophulariiflora* (originally written as "*P. scrophulariaeflora*"). The genus *Picrorhiza* and the species *P. kurrooa* appeared for the first time on a drawing published by Royle on 24 August 1835 (Stafleu and Cowan 1983) in his "Illustrations of Botany" (Fig. 2; Royle 1835a). Bentham described the genus and the species in his "Scrophularineae Indicae", which was published a few months later, on 17 November 1835 (Bentham 1835). However, he wrote the species name as "*P. kurroa*", probably correcting or misspelling the name of Royle's publication. In May 1836, Royle published the text on *Picrorhiza*, and retained the species name "*P. kurrooa*" (Royle 1835b). Because, Bentham was the first to give a written description of the species, the accepted species name has been "*Picrorhiza kurrooa* Bentham", or alternatively "*Picrorhiza kurrooa* Royle ex Bentham". However, according to the International Code of Botanical Nomenclature article 42, an illustration with analysis published before 1 January 1908 is acceptable as valid publication, instead of a written description or diagnosis (Greuter et al. 1994). Therefore, the correct name of the species is "*Picrorhiza kurrooa* Royle" (Smit 2000). Royle (1835b) states that the generic name is derived from the bitter root, which is used in native medicine. In Greek, "picros" means bitter, while "rhiza" means root. The specific name is derived from "Karu", the Punjabi name of the plant, which means bitter as well (Coventry 1927). In 1876, Bentham and Hooker described the genus *Picrorhiza* in their "Genera Plantarum", mentioning the existence of only one species. The genus was

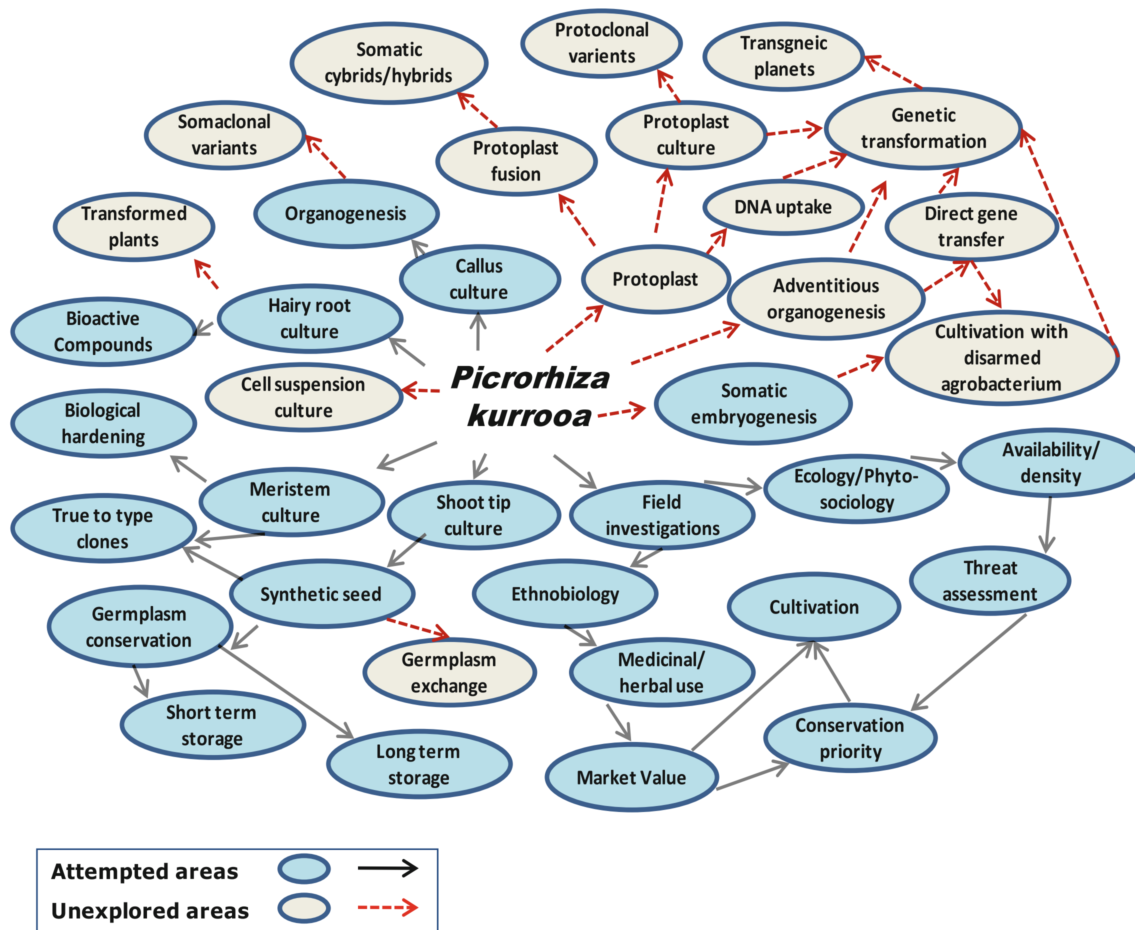

Fig. 1 Extent of research on ecological and biotechnological interventions in *P. kurrooa*

considered monotypic until Pennell distinguished another species, based on information in Hooker's "Flora of British India" (Pennell 1943). Hooker considered the flowers of *P. kurrooa* as dimorphic, which was so far not known in the Scrophulariaceae. He distinguished a form with long stamens and a short corolla with five sub-equal lobes, and a form with short stamens and a bilabiate corolla, of which the upper lip is longer and the lower lip consists of three shorter lobes (Hooker 1885). Other important characteristics of *P. kurrooa* especially the phenological and taxonomic details are beautifully explained by Smit (2000).

Picrorhiza kurrooa is an important medicinal plant endemic to alpine Himalaya (Thakur et al. 1989), distributed from Kashmir to Kumaon between 2,800 and 4,800 m amsl (Fig. 3). The flowers have two pairs of anther-bearing stamens, and a sterile fifth stamen—a taxonomically important feature. All these parts are attached at the base of the ovary. The flowers are bisexual and sometimes have brightly coloured and conspicuous-associated bracts. Flowers, which appear in June through August, are white or pale purple and borne on a tall spike. The leaves are 5–10 cm long, alternate, opposite, or sometimes whorled, and are simple to pinnately divide. The fruit type is usually a 2-chambered capsule. It has a long, creeping rootstock that is bitter in taste, and grows in rock crevices and moist, sandy soil. Manual harvesting of the plant takes place in October through December. Runner of the plant contains several important iridoid glycosides, e.g., kutkoside, picroside I, picroside II, picroside III and picroside V. These iridoids have wide range of biological activity such as cardiovascular, antihepatotoxic, choleretic, hypoglycemic, hypolipidemic, antiinflammatory, antispasmodic, antitumor, antiviral, purgative, immunomodulatory, antioxidant, anti-phosphodiesterase, neuritogenic, molluscicidal, and leishmanicidal activities (Hussain 1984; Kirtikar and Basu 1984; Ansari et al. 1988; Joy et al. 2000). Extensive research has been devoted to standardized iridoid fractions of *P. kurrooa*, e.g. Kutkin and Picroliv. Kutkin is mixture of the glucosides picroside I and kutkoside in a ratio of 1:2 and other minor glycosides (Singh and Rastogi 1972; Ansari et al. 1988), whereas, Picroliv is very important but less purified mixture of picroside I and kutkoside (1:1.5 ratio; Dwivedi et al. 1989; Dhawan 1995).

Fig. 2 First publication on *Picrorhiza kurrooa* by Royle in 1835 (*source*: Smit 2000)

Number of publications referred the importance of Picroliv as antihepatotoxic, hepatoregenerative, choleretic, and hypolipidemic. Furthermore, Picroliv enhanced non-specific immune responses of peritoneal macrophages and the [3H]-thymidine uptake by lymphocytes isolated from mice treated with Picroliv (Puri et al. 1992).

Ecological status

A few studies on ecological status of *P. kurrooa* has been performed in last few years (Uniyal et al. 2000; Arya 2002; Joshi 2002; Nautiyal et al. 2002, 2003; Kala 2005; Singh et al. 2007; Semwal et al. 2007). As compared to other species in the same natural habitat/landscape units, density (individuals/m^2) of *P. kurrooa* was found very low. A comparative account on the availability of five highly demanded medicinal plants of Himalaya which are also listed in top 20 medicinal plants traded in India (Nag 2011) including *P. kurrooa* is presented in Table 1. Keeping in mind its uses in curing various ailments, increasing demand in the industries and consequently its less availability in natural habitats, sustainable cultivation of this species have been highlighted in last few years (Anonymous 2003; CUTS 2004; Kala et al. 2006; Nag 2011). Limited progress has been achieved to protect and sustain this species in its natural habitats as well as in cultivated lands for future. Due to indiscriminate collection from the wilds and on account of the absence of organized cultivation, this plant is listed as 'endangered' (Nayar and Sastry 1987; Samant et al. 1998; Rai et al. 2000). Being included in the negative list of Indian plants[by Ministry of Commerce, GOI, Vide Notification No. 03 (RE-2003)/2002–2007 (Appendix II) dated 31st March 2003 issued by Director General of Foreign Trade,

Fig. 3 Distribution of *Picrorhiza kurrooa* Royle in the Himalaya region (*source with modification*: Smit 2000)

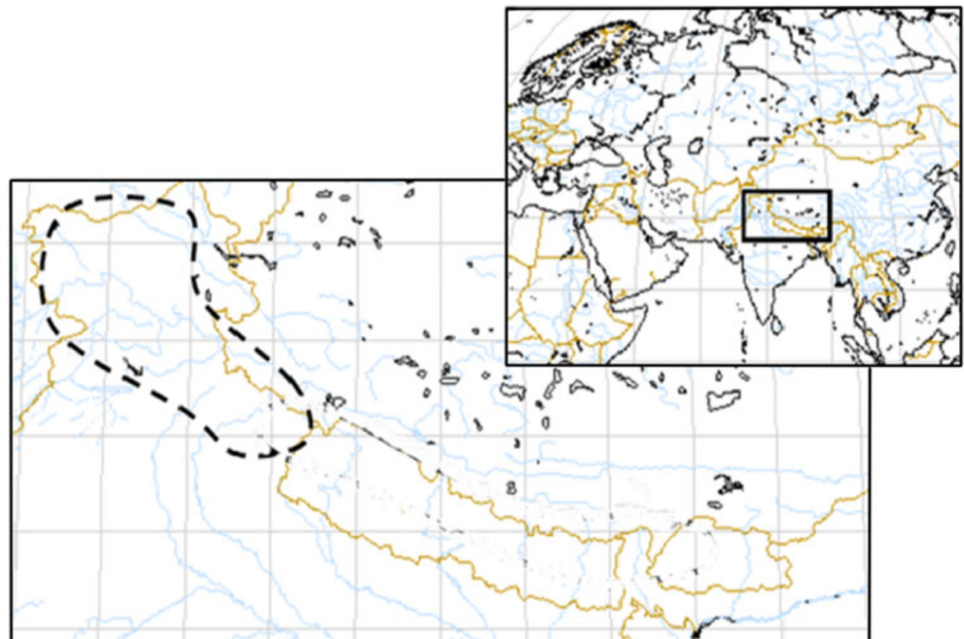

Table 1 Comparative account on availability/density (individuals/m^2) in nature of important Himalayan medicinal plants listed in top 20 medicinal plants traded in India

Species	NW Himalaya		Protected areas of NW Himalaya	Threat
	KH, GH etc.	ITH	NDBR, VOF, KWLS etc.	RDB, IUCN
Aconitum heterophyllum Wall. ex Royle	0.48[a], 1.40[b,c]	1.00[d]	3.60[f], 0.060[h]	CR[i,l]
Dactylorhiza hatagirea (D. Don) Soo	0.48[a]	16.20[a]	4.20[a], 1.00[a], 3.50[f], 0.08[g], 0.03[h]	CR[i,j]
Picrorhiza kurrooa Royle ex Benth.	3.90[a]	11.40[d], 0.07[a]	4.50[a], 3.50[f]	EN[i,d,j,k]
Aconitum violaceum Jacq.	–	–	2.10[e]	VU[i,j]
Nardostachys grandiflora DC.	1.70[a]	–	0.80[e]	CR[i]

NW north west, *KH* Kumaun Himalaya, *GH* Garhwal Himalaya, *ITH* Indian Trans Himalaya, *NDBR* Nanda Devi biosphere reserve, *VOF* valley of flowers; *KWLS* Kedarnath wildlife sanctuary, *RDB* red data book, *IUCN* International Union for Conservation of Nature and Natural Resources, *CR* critically endangered, *EN* endangered, *VU* vulnerable

[a] Uniyal et al. (2000), [b] Nautiyal et al. (2002), [c] Nautiyal et al. (2003), [d] Singh et al. (2007), [e] Kala (2005), [f] Semwal et al. (2007), [g] Joshi (2002), [h] Arya (2002), [i] Ved et al. (2003), [j] Srivastava et al. (2010), [k] Kala et al. (2004), [l] Malik et al. (2011)

Govt. of India] export of *P. kurrooa*, in any form, is not permitted by the Export Authority of India (Purohit 1997).

Tissue culture-mediated biotechnological interventions

Seed germination

Regeneration from seeds is the most commonly used method of propagation in many plant species (Sharma et al. 2006). Like many other alpine species, seeds of *P. kurrooa* in nature are subjected to chilling at subzero temperatures during winters which may assist germination of the seeds. Nautiyal (1988) reported up to 60 % germination in *P. kurrooa* by incubation of seeds at 20 °C in light, while Chandra et al. (2006) reported poor germination in freshly harvested seeds of *P. kurrooa*. This is possibly due to dormancy, exhibited by many alpine seeds as an adaptation to overcome the harsh climatic conditions prevalent at high altitudes (particularly after seed shedding in October), where naturally controlled germination can be crucial to seedling survival (Kaye 1997; Nadeem et al. 2000; Pandey et al. 2000). In a soil-based experiment inside a polyhouse, up to 58 % seed germination was observed without any chemical treatment when seeds were sown in sandy loam soil covered with moss (Nautiyal et al. 2001). Chandra et al. (2006) also reported that irrespective of the germination temperature, presoaking treatment with GA_3 significantly improved percent germination (above 80 %) and reduced the time required for germination. Germination of various herbaceous species, including those of alpine regions, is known to be accelerated by the treatment of seeds with GA_3 or BAP, or a combined treatment with both (Son et al. 1999; Nadeem et al. 2000; Pandey et al. 2000).

Jan et al. (2010) observed that all the seeds treated with different temperatures showed germination in petriplates. Maximum 93 % germination reported at 4 °C (cold treatment; 5–10 days) on absorbent cotton provided with 16 h photoperiod. This suggests that lower temperature had some effect on the germination rate and could be altering the functions of cold-shock proteins.

Vegetative propagation

Clonal propagation using 'vegetative means' provides a simple method for multiplication of *P. kurrooa*. Nearly 87 % rooting has been achieved when runner cuttings were treated with IBA or NAA, either alone or in combination with BAP (10.0 μM; Chandra et al. 2006). In an earlier study, more than 90 % rooting of apical segments taken from runners has been reported, however, rooting in basal cuttings was poor (39 %; Nautiyal et al. 2001).

In vitro propagation

In vitro propagation techniques have been widely used for the commercial propagation of a number of economically important species (Bhojwani and Razdan 1996; Ravishankar and Venkatraman 1997). This technique is useful not only for rapid and mass multiplication of existing stock of plant species but also offer advantages for the conservation of important and threatened/endangered taxa. The composition of nutrient medium, concentration and permutation–combination of plant growth regulators and environmental ambience in culture, genotype and physiological status of the donor plant play a critical role in micropropagation. In addition, the success of micropropagation depends on the selection of a suitable plant part to serve as the starting experimental material, i.e. explant.

Shoot multiplication through nodal explants/shoot tips

Study by Lal et al. (1988) was perhaps the earliest report on tissue culture of *P. kurrooa* using shoot tips as explant taken from natural plant maintained under controlled conditions. They found that MS medium supplemented with kinetin (3.0–5.0 mg/l) supported rapid proliferation of multiple shoots from the explants. Addition of IAA (1.0 mg/l) to the Kn containing medium showed remarkable improvement in the growth of regenerants. Subsequently, Upadhyay et al. (1989) reported in vitro propagation and vitrification of shoots in cytokinin-supplemented medium. The application of BAP at lower concentrations (1.0 μM) has proven extremely beneficial for induction of multiple shoots and subsequent shoot multiplication (Lal et al. 1988; Upadhyay et al. 1989). Chandra et al. (2004) also reported that higher concentration of BAP (2.5–5.0 μM) 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 (Chandra et al. 2004; Upadhyay et al. 1989).

Sood and Chauhan (2009a) have developed a low cost micropropagation technology for this medicinally important herb *P. kurrooa*. Axillary shoot tips cultured on MS medium supplemented with 2 mg/l IBA and 3 mg/l Kn were found the best with 86.3 % shoot apices proliferating into multiple shoots. The sucrose was replaced with table sugar and agar was omitted completely. Out of six low cost media combinations tested, MS liquid media supplemented with IBA (2 mg/l) + Kn (3 mg/l) + table sugar 3 % (w/v) were found to be the best with 27 shoots/explant. Seventy percent shoots formed roots on half strength MS salts supplemented with IBA (3 mg/l) + table sugar 3 % (w/v) + agar with an average of 5.6 roots per shoot.

Cotyledonary node

Cotyledonary node is an alternative explant for in vitro propagation. Shoot multiplication through cotyledonary node has been reported by Chandra et al. (2004). They used cotyledonary stage seedlings before the onset of growth and visible appearance of the plumule to obtain cotyledonary node explants. They found that such cotyledonary node explants from in vitro grown seedlings have shown limited competence. Maximum shoot multiplication was observed on MS medium supplemented with 1.0 μM BAP or Kn (Chandra et al. 2004).

Axillary buds

In vitro shoot multiplication through sprouting of axillary buds using nodal segment has been reported (Chandra et al. 2006). The axillary shoots were obtained from nodal segments after 2 weeks of inoculation on MS basal medium supplemented with 1.0 μM BAP. Multiple shoot formation started within 3 weeks in 66 % of cultured shoots. The average number of shoots per cultured axillary shoot was recorded to be 9.2 after 4 weeks of culture with a maximum of 14 shoots from a single axillary shoot.

Callus culture and direct/indirect organogenesis

Callus culture (Fig. 4a) is a good alternative source for production of in vitro plants. Lal and Ahuja (1995) reported the maintenance of shoot and callus cultures of *P. kurrooa* for 64 weeks on hormone-free MS medium incubated at 4–10 °C in the dark. Later they (Lal and Ahuja 1996) have reported induction of callus from nodal cuttings and leaf explants of *P. kurrooa* on MS medium supplemented with 0.5–2.0 mg/l 2,4-D. Maintenance and proliferation of callus were optimal on MS medium containing 4.0 mg/l NAA and 1.0 mg/l Kn. Shoot organogenesis was achieved from callus by its sequential transfer at different media, i.e. induction of bud primordial on MS medium with 0.25 mg/l BAP and conversion of bud primordia into shoots on MS + 0.12 mg/l BAP (with half strength nitrogen supply). Single combination of media was not found suitable for shoot bud/somatic embryo formation in this species. Regenerated shoots were rooted on MS medium containing 0.2 mg/l NAA and further successfully established in soil (Lal and Ahuja 1996).

Sood and Chauhan (2009b) established callus cultures from different explants of *P. kurrooa* such as leaf discs, nodal and root segments of *P. kurrooa*. Callus induction was highest (70 %) in root segments followed by leaf discs (56.3 %) and nodal segments (38.3 %) on MS medium supplemented with 2,4-D (2 mg/l) and IBA (0.5 mg/l). The callus cultures derived from different explants were differentiated into multiple shoots on MS medium containing different concentrations and combinations of BA, Kn and IBA. Regeneration was highest in the calli derived from root segments and leaf discs on MS + BA (2 mg/l) and Kn (3 mg/l) with 76.7 and 72.2 % calli forming shoot primordia, respectively. Most of the nodal segments-derived calli got differentiated into roots rather than shoots.

Jan et al. (2010) reported callus formation in *P. kurrooa* on MS medium supplemented with different concentrations of 2,4-D and direct regeneration was found with BAP. They found that MS medium with 0.25 mg/l 2,4-D and 0.25 mg/l BAP stimulated callus induction rate and direct shoot regeneration rate. Jan et al. (2010) reported indirect and direct adventitious shoot regeneration, multiplication as well as their elongation on MS medium using various concentrations of NAA (0.2, 0.4, 0.6, 0.8 and 1.0 mg/l) alone or in combination with IAA and IBA within 10 week culture for the first time. Earlier, Upadhyay et al. (1989) reported in vitro propagation using BAP, however,

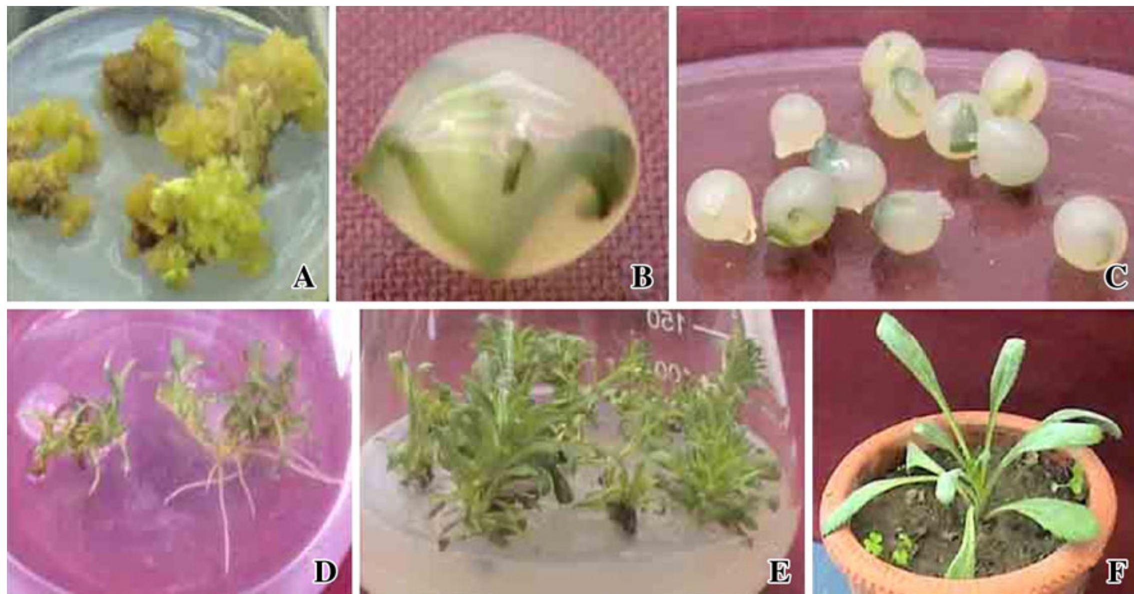

Fig. 4 **a** Callus culture of *P. kurrooa*, **b**, **c** encapsulated microshoots, **d** shoot proliferation and simultaneous rooting from synthetic seed, **e** well grown in vitro plants, **f** hardened plant in green house

according to Jan et al. (2010), BAP has not been proven effective for in vitro propagation. These variations in the response of *P. kurrooa* could be due to ecotypic differences (Mohapatra and Greshoff 1982). The average shoot number 18.4 per explant for indirect organogenesis was observed on 0.2 mg/l NAA with 80 % response and 18.3 per explant for direct organogenesis was observed on 0.6 mg/l NAA with 95 % response (Jan et al. 2010).

There is no published report of somatic embryogenesis in *P. kurrooa*. However, Bantawa et al. (2009, 2011) established protocols for micropropagation, somatic embryogenesis and organogenesis of *P. scrophulariiflora* (the only second species in genus *Picrorhiza*).

Table 2 represents the various reports on in vitro culture of *P. kurrooa* using various plant parts as explants, e.g., leaf discs, cotyledonary node, shoot tips, nodal segment, root segments, etc. on Murashige and Skoog (1962) medium.

Rooting of tissue culture raised plant

Rooting is an important step for developing a complete in vitro propagation protocol. Successful rooting of microshoots in *P. kurrooa* can be achieved by incorporation of various auxins (NAA, IBA and IAA) in the culture medium (Lal et al. 1988; Upadhyay et al. 1989). Although, Lal et al. (1988) did not mention the rooting percentage, Upadhyay et al. (1989) achieved 89 % rooting in microshoots with 1.0 μM NAA in 20 days. Chandra et al. (2006) reported 100 % in vitro rooting of microshoots with 1.0 or 2.5 μM IBA.

Sood and Chauhan (2009a, b) observed root induction in 9–10 days of culturing with highest 70 % rood induction frequency on MS medium containing IBA (3 mg/l) followed by 66.6 % on MS supplemented with IBA (3 mg/l) and NAA (2 mg/l). The same medium was found suitable for more number of roots/shoot and maximum root/shoot length. Jan et al. (2010) observed direct root initiation and elongation after sub culturing of isolated, elongated shoots onto MS basal medium supplemented with different concentrations of NAA (0.0, 0.2, 0.4, 0.6, 0.8 and 1.0 mg/l) alone as well as in combination with IAA and IBA (0.1, 0.25 and 0.5 mg/l each) within 4 weeks of their culture period. The rooting response of shoots was 92 % in MS with 0.4/mg NAA. The primary roots become visible after 7 days of culturing, however, long thin multiple roots were achieved after 4 weeks (Jan et al. 2010).

Hardening of tissue culture raised plants

Hardening is one of the most crucial part of plant tissue culture in which in vitro raised plants get acclimatize to the external environment. Chandra et al. (2006) reported 65 % survival of the rooted plants transplanted in thermocol cups containing a mixture of soil and sand (1:1) and hardened under high relative humidity (80.0 ± 5.0 %) for 6 months in the greenhouse. In another study, Jan et al. (2010) reported 81.5 % survival of complete plantlets hardened in polycups containing sterile soil, sand and vermiculite (1:1:1). Well-rooted plantlets were transferred to pots containing autoclaved potting mixture consisting of sand, soil and vermiculite (1:1:1) in the greenhouse for hardening (Sood and Chauhan 2009a, b). During the hardening process, glass beakers were taken off every day for 1–2 h so as to acclimatize the plantlets to external environment.

Table 2 A detail account of work carried out on in vitro propagation of *Picrorhiza kurrooa* in last two decades

Explant used	Media + plant growth regulator			Result	References
	Callus induction/ proliferation	Shoot multiplication	Rooting		
Shoot tip from natural plant maintained in controlled condition	–	MS + Kn 3–5 mg/l, MS + IAA 1.0 mg/l + Kn 3.0 mg/l	MS + NAA 1.0 mg/l	Shoot proliferation and rooting	Lal et al. (1988)
Terminal and single node cutting from natural plant	–	–	MS + 1.0 µM NAA	Shoot induction and rooting	Upadhyay et al. (1989)
Shoot tip from in vitro grown plant	MS without plant growth regulators	MS without plant growth regulators	–	Callus and shoot maintenance	Lal and Ahuja (1995)
Leaf and nodal cuttings from natural plant	MS + 0.5–2.0 mg/l 2,4 D, 4.0 mg/l NAA, 1.0 mg/l Kn	MS + 0.25 mg/l BAP, MS + 0.12 mg/l BAP	MS + 0.2 mg/l NAA	Callus, shoot induction and rooting	Lal and Ahuja (1996)
Cotyledonary node and shoot tips from in vitro grown seedlings	–	MS + 1.0 µM BAP	MS + 1.0 or 2.5 µM IBA	Plantlets and rooting	Chandra et al. (2004)
Nodal segments of natural plant	–	MS + 1.0 µM BAP	MS + 1.0 or 2.5 µM IBA	Multiple shoots and rooting	Chandra et al. (2006)
Axillary shoot tips from potted plants	–	MS + 2 mg/l IBA + 3 mg/l Kn	MS + 3 mg/l IBA	Multiple shoots and rooting	Sood and Chauhan (2009a)
Leaf discs, nodal segment and root segments of in vitro grown plant	MS + 2 mg/l 2,4 D + 0.5 mg/l IBA	MS + 2.0 mg/l BA + 3 mg/l Kn	MS + 3.0 mg/l IBA	Callus, plant regeneration and rooting	Sood and Chauhan (2009b)
Nodal segment from germinated seed and mature plant	MS +0.25 mg/l 2,4 D, MS + 0.25 mg/l BAP	MS + 0.2 mg/l NAA, MS + 0.6 mg/l NAA	MS + 0.4 mg/l NAA	Callus, direct and indirect organogenesis and rooting	Jan et al. (2010)

Biological hardening

Successful hardening and post-hardening establishment of tissue culture-raised plants in their natural habitat is an important step for propagation of medicinally and commercially valuable species. Trivedi and Pandey (2007) used three plant growth-promoting rhizobacteria viz. *Bacillus megaterium*, *B. subtilis* and *Pseudomonas corrugata* for biological hardening of micropropagated plantlets of *P. kurrooa*. Inoculation of micropropagated plants by the three bacterial isolates was effective in improving the survival of plants after transfer to soil. Maximum survival (94.5 %) was observed in plants inoculated with *B. megaterium*. The survival percentage was 92.5 and 85.0 % in plants inoculated with *B. subtilis* and *P. corrugata*, respectively, while only 38.5 % survival was observed in control plants. The bacterial inoculations also influenced plant growth positively in terms of plant growth parameters and phosphorus content (Trivedi and Pandey 2007). Earlier, Chandra et al. (2004) reported that soil inoculated with known antagonistic bacteria, *B. subtilis* and *P. corrugata*, was found to be better for plant survival during hardening in the greenhouse.

Cryopreservation and synthetic seed production

Cryopreservation of shoot tips of *P. kurrooa* through vitrification has been reported by Sharma and Sharma (2003). About 1 mm long segment of shoot tips were precultured on MS medium supplemented with various osmotic. Further, these shoot tips were successfully cryopreserved by vitrification, and precultured on medium supplemented with 5 % DMSO at 4 °C. Average survival was about 20 % in terms of normal shoot formation after 4 weeks. Sharma and Sharma (2003) reported significant improvement in the survival and shoot regeneration of cryopreserved shoot tips (70 and 35 %, respectively).

Synthetic seed can be considered as “genetically identical materials”. The potential advantages of synthetic seed include ease of handling and transportation along with increased efficiency of in vitro propagation in terms of space, time, labor and overall cost (Nyende et al. 2003).

The conservation of germplasm of *P. kurrooa* through encapsulated microshoots has been reported recently (Mishra et al. 2011a). Microshoots were encapsulated in a combination of 3 % sodium alginate, 1.5 % (w/v) solution of sucrose and 3 % (w/v) calcium chloride solution ($CaCl_2$; Fig. 4b). The encapsulated microshoots were stored in a moist-environment in a flask for 3 months at 25 ± 2 °C; moist conditions were maintained by spraying encapsulated shoots with sterile distilled water (in the form of mist) at 15 days interval (Fig. 4c). Following storage, the encapsulated microshoots were placed in half strength MS basal medium for regrowth (Fig. 4d). Mishra et al. (2011a) have recorded regrowth frequency as well as the number of plantlets that developed both shoots and roots after 2, 3 and 4 weeks of culture. The success rate of regrowth was found to be approximately 90 % following 3 months of storage. Of the encapsulated microshoots, 42.66 % exhibited formation of multiple shoots following regrowth on plant growth regulator free MS medium (Fig. 4e). Developing shoots without roots were further transferred to half-strength MS medium supplemented with 1.0 μM NAA for 100 % rhizogenesis. Healthy root formation was observed in all microshoots following 2 weeks of transfer and they were transferred to green house (Fig. 2f).

Assessment of genetic fidelity

The most important objective of tissue culture is to achieve increased plant yield without compromising quality. However, in an increasingly competitive market, both increase in yield and betterment in quality of the plant are required. Thus, for mass scale production, efficient, reliable and cost-effective propagation methods along with long-term genetic stability of plantations are of pivotal importance. Although evaluation of clonal stability is difficult and time consuming, ensuring genetic fidelity (stability) is essential, particularly in tissue culture raised populations.

Various markers, e.g. morphological, physiological and biochemical, have routinely been used to characterize plant material. Although these descriptors are valuable for identification of the plant and can reveal, to some extent, inter and intra varietal polymorphism, they can not account for the total diversity in the tissue culture raised plants. Therefore, application of DNA-based markers like random amplified polymorphic DNAs (RAPDs), inter simple sequence repeats (ISSRs), single nucleotide polymorphisms (SNPs), cleaved amplified polymorphic sequence (CAPS), simple sequence repeats (SSRs), restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), etc. provide more reliable tools for the determination of genetic variability.

The genetic fidelity of plants recovered following cryogenic storage has been assessed in a wide range of plant systems (Aronen et al. 1999; Hirai and Sakai 2000; Bekheet et al. 2007). The genetic fidelity of *P. kurrooa* plants, developed after the storage of encapsulated microshoots, was assessed through RAPD analysis (Mishra et al. 2011a). Cluster analysis of the RAPD profile revealed an average similarity coefficient of 0.966 confirming genetic stability of plants derived from encapsulated microshoots following 3 months of storage at 25 °C. The results further support the feasibility of this cost effective and simple germplasm conservation approach for storage and regeneration of true-to-type plants.

Genetic transformation

The role of *Agrobacterium rhizogenes*-mediated hairy root cultures as an efficient production alternative has undeniably proved its effectiveness in the worldwide arena (Guillon et al. 2006a, b; Hu and Du 2006). Advantages of hairy roots include rapid biomass accumulation, typically accompanied with a high production of secondary metabolite and the possibility for upscaling in specialized bioreactors (Kim et al. 2002; Georgiev et al. 2007). Hairy roots have been established to genetically manipulate and evaluate the production of secondary metabolites in *P. kurrooa* (Verma et al. 2007; Mishra et al. 2011b).

Verma et al. (2007) developed a protocol for induction and establishment of hairy root through *A. rhizogenes* using leaves and stem segments of *P. kurrooa* as explants. They observed 66.7 % relative transformation frequency after the infection of leaf with bacterial strain LBA9402. The emerging hairy roots were cultured to the half and full strengths of the B5 medium containing 3 % (w/v) sucrose for root proliferation. The whole study was carried out with nine hairy root clones. Higher amounts of secondary metabolites (kutkoside and picroside I) were reported in all the nine clones in comparison to non-transformed, in vitro-grown control roots of *P. kurrooa*. In another study, Mishra et al. (2011b) reported hairy roots of *P. kurrooa* following co-cultivation of shoot tip explants (Fig. 5a) with *A. rhizogenes* strains A 4 and PAT 405. Confirmation of transformation was done by PCR analysis (Fig. 5b). They found that bacterial strain A 4 was better than the strain PAT 405 in terms of both growth of respective hairy root cultures and secondary metabolite production. Picrotin and picrotoxinin from the roots of wild-type field grown plants were compared with 8-week-old hairy root cultures induced by the A 4 and PAT 405 strains of *A. rhizogenes*. In terms of the production of picrotin and picrotoxinin, the A 4 induced hairy roots appeared to be a better performer than the PAT 405 induced hairy root cultures. The picrotin and picrotoxinin content was highest in 8-week-old A 4

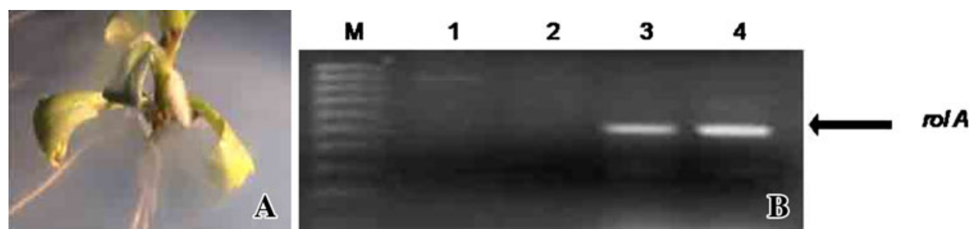

Fig. 5 **a** Hairy root induction from the nodal region of *P. kurrooa*, **b** confirmation of transformation of hairy roots through PCR analysis

induced hairy roots (8.8 and 47.1 µg/g DW, respectively). These works undoubtedly highlights the significance of the present research endeavor, which can endow us with a substitute for the exploitation of this commercially important endangered, hitherto unexplored medicinal plant species.

Conclusions and future prospects

Till date, very slow progress has been made regarding the development of rapid, reproducible and reliable in vitro plant regeneration systems for *P. kurrooa*. At the same time, the cultivation approaches adopted for conservation and production of *P. kurrooa* are neither progressive nor profitable due to the lack of awareness and interest of the farmers as well as other stakeholders, supportive government policies, availability of assured markets, profitable price levels, and assess to simple and appropriate agro-techniques. Therefore, tissue culture-mediated biotechnological interventions, e.g. in vitro multiplication, callus culture, hairy root culture, etc., can lead to development of effective way for plant preparation and can be helpful for reducing pressure on the natural plant population.

In conclusion, medicinal plants are the area which requires concerted efforts and for which the world demand continues to increase. There is a need for improved techniques of propagation to rapidly supply large number of plants. The synthetic seed technology involving nutrient alginate encapsulation developed for *P. kurrooa* could be useful in germplasm distribution and exchange. The most important part of any in vitro propagation system is mass multiplication of plantlets which are phenotypically uniform and genetically akin to the mother plant, otherwise the advantage of desirable characters of elite/supreme clones will not be achieved. Estimation of genetic fidelity of micropropagated plants is also an important research task. If done properly, micropropagation of selected suitable clones is poised to make spectacular contribution to the growth of pharmaceutical industry.

The ever increasing demand of alpine medicinal herbs of the Indian Himalayan region (IHR) as source of active compounds of modern medicines has resulted in depletion in their natural habitat. *P. kurrooa* is a high value medicinal plant and thus this species is facing threat due to excessive and illegal collection from the wild. Hairy root cultures have proven to be an efficient means of producing secondary metabolites that are normally biosynthesized in roots of plants. It must be mentioned that so far there are no reports on regeneration of plants through hairy roots in *P. kurrooa*. Based on these preliminary findings, hairy root cultures can be a potential alternative source of secondary metabolites. However, sometimes the efficiency of secondary metabolite production is not so desirable. Metabolic engineering offers new perspectives for improving the production of secondary metabolites by the over expression of single genes. This approach may lead to increase in some enzymes involved in metabolism and consequently results in the accumulation of the target products. In essence, biotechnological interventions have opened up new vistas for genetic improvement of this medicinally important plant.

Author contribution Being the first author, Balwant Rawat has written the whole manuscript. He also has prepared the whole ecological part in the manuscript. Janhvi Mishra Rawat is expert in current biotechnological aspects and has prepared the overall biotechnological part in the manuscript. Susmita Mishra is working in the field of genetic engineering since last few years and has contributed the genetic transformation part in the manuscript. Being the senior most author, Dr. Shri Nivas Mishra has developed the concept and frame of the manuscript. The manuscript was polished time to time by Dr. Mishra.

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