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REVIEW ARTICLE

Picrorhiza kurroa Royle ex Benth: potential of hairy root cultures for bioactive metabolic principles

Bhawna Pandey[1] · A. K. Gaur[1]

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Abstract
Picrorhiza kurroa plantation varies from subalpine to alpine ranges 3000-5300 m (msl) observed extensively across North Western to North Eastern Himalaya. International Union for Conservation of Nature and Natural Resources (IUCN) has already listed *P. kurroa* and related species under endangered flora. Bioactive leads, primarily two major principle(s) picroside I and picroside II besides many others, are derived from metabolic pathways especially in the underground roots cum rhizomes after 3–4 years growth of this plant. Bioactive principles have been validated to revive vital function as evident from large demand in local markets. The growing demand for plant derived chemicals as such in various pharmaceutical industries has threatened the survival of many such endangered plant species. Researchers are developing alternatives for large scale production protocols from laboratory scale, medium scale, large scale and commercial scale production of such bioactive metabolites. *Agrobacterium rhizogenes* (*Rhizobium rhizogenes*) are virulent strains that often develop hairy roots. In vitro protocols are quite effective while growing co-cultivated explants to develop hairy roots. Since hairy roots potential for bioactive principles has been well documented, therefore, this particular review aims to highlight recent progress emphasizing feasibility and further potential applications in developing commodities for local and global markets.

Keywords *Agrobacterium rhizogenes* · Bioactive principles · Kutki · Pharmaceutical · Picrosides · *Rhizobium rhizogenes*

Introduction

It has generally been observed that endangered plants of the Himalaya that mostly (95%) are still being collected from the wild to meet out the requirement of most of the pharmacy (Chandra 2016). *Picrorhiza kurroa* Royle ex Benth has emerged locally as the most promising plant under cultivation through contract farming. Such farming practices paying rich dividends to local community leading to their prosperity. Quality propagules have proven potential for marketing by virtue of retaining the active components picroside I, picroside II, Kutkoside and many more beside sustainable practices for the germplasm (Nautiyal and Nautiyal 2004). Since ancient time tribal communities in Chamoli, Garhwal maintained *Picrorhiza kurroa* without much of gains in terms of livelihood therefore this particular plants comes under community plants. Recently these communities have begun cultivating this plant commercially with scientific inputs leading to income generation upto a larger extent and providing employment opportunities (Chand et al. 2016). Growers organization at Ghaise (a remote village in Garhwal) situated in Deval block alongwith public–private partnership presents a suitable case study in collaboration with an International firm and the High Altitude Plant Physiology Research Centre (HAPPRC) affiliated with Hemvati Nandan Bahuguna Garhwal University, Srinagar, Garhwal (Uttarakhand) (Bisht et al. 2011). Thus, cultivation of Kutki in central Himalaya is contributing to the agricultural economy through prospering regional community at least upto moderate level resulting in enhanced livelihood. Plant (Kutki) parts such as leaves, roots/ rhizome including entire plant as such are frequently collected illegally from the forest (Masood et al. 2015). Raising tissue cultured plantlets even without genetic modification takes care at least the origin of plantlets selecting the mother plants required for micro propagation as a step zero. Bioactive

✉ Bhawna Pandey
58482@gbpuat.ac.in; pandeybhawna046@gmail.com

[1] Molecular Biology and Genetic Engineering, College of Basic Sciences and Humanities, Govind Ballabh Pant University of Agriculture and Technology, Pantnagar, Udham Singh Nagar, Uttarakhand 263145, India

metabolites from wild habitats of the plants are dependent on climate change behavior resulting into multiple environmental factors leading to undesirable bioactive principles (Applequist et al. 2020). Since, commercial value of *Picrorhiza kurroa* has already established which has led more concerns about its sustainable harvest and conservation strategies. Reported literature emphasized that several plant biomolecules are derived in infectious roots from virulent strains of bacteria such as Rhizobium and or Agrobacterium species/ strains. One such example is hairy roots which develop after such infections where activity of metabolic principles and their production reached at a more beneficial level of their biogenesis (Georgiev et al. 2007). Different in vitro culture including plant cell/tissue cultures, bioreactor cultivation are under practice since 1950s (Giri and Narasu 2000; Cui et al. 2011). Biogenesis of bioactive principles (picroside I, picroside II, kutkoside, picrotin and picrotoxinin) since triggered by elicitation through desired elicitor(s), beside precursor feeding for a desired product in in vitro cultures have been found to facilitate many of times fair production levels of required lead molecules (Biswas et al. 2023). Therefore, we intend that much of studies though had been reported in the scientific literature but hairy roots detailed observations in terms of field grown/ greenhouse grown/in vitro grown even during bioreactor parameter optimization with inoculated hairy roots are not much defined. Thus, our aim is to review and mining valuable information which in turn would be beneficial to those who are optimizing various strategic steps of proceeding toward maximum benefit efficiently. Figure 1.

In view of the described facts, we accessed the availability of *Picrorhiza kurroa* (kutki) from grower's field in the central Himalaya. Plants were collected from Jhuni, Bageshwar in the Northwestern Himalayan region of Uttarakhand, India. These plants were growing at an elevation of 520 m (1706.04ft) at coordinates 29.94^{o}N 79.90^{o}E, propagating through rhizome. During rearing of kutki plants from grower's fields these were maintained at green house facilities grown in pots containing soil: sand: compost in the ratio of 1:1:1(w/w/w) at an elevation of 243.8 m (799.8ft) and coordinates 29^{0}N 79^{0}E as well as under laboratory in vitro practices.

Hairy root genesis

Stolon cuttings propagation

Vegetative propagation from stolon segments is effectively achieved with water-dip irrigation practices for upto 48 h. Stolon cuttings facilitate while used in trenches in order to retain more moisture in the soil. This approach is easily practicable for local growers especially when planting material is available out of season. Stolon top segments are found more suitable for multiplication. Rooting in these top segments is typically observed after two to three weeks (Nautiyal et al. 2001).

Fig. 1 *Picrorhiza kurroa* grown in pots containing soil (collected from Dr NE Borlaug Crop Research Centre, Pantnagar-263145): sand: compost (obtained from cow dung collected from Dairy Research Farm) in the ratio of 1:1:1 w/w/w

Intercropping system

Intercropping of *Picrorhiza kurroa* with other suitable beneficial plants has been successfully observed with *Foeniculum vulgare*, *Digitalis purpurea* and *Solanum tuberosum*. Among these intercropping observed more beneficial with *Foeniculum vulgare* by virtue of enhanced production. *F. vulgare* creates amicable microclimate to growing plants of *P. kurroa* through retaining moisture due to canopy for a long time (Nautiyal et al. 2001). Soil with higher forest litter also promotes improved growth and yield of kutki. At lower altitudes, plants are susceptible to powdery mildew which could be managed by spraying Topsin-M (thiophinate methyl 0.1%) about 15–20 days after onset of infection followed by second application after 15 days. During flowering and seed formation, spraying of insecticide (ecalux, 0.5%) twice at 10-day intervals found to prevent further loss of growth and development due to insects and aphid infestations (Chand et al. 2016).

Greenhouse growth pattern

Field grown plants of 8–10 cm height with average rhizome length of 6 cm were nurtured and maintained in pots of (2L) with drainage holes consisting soil: sand: compost in the 1:1:1 (w/w/w) ratio. Powdery mildew stress was observed and managed by foliar application of 0.2% (w/v) sulfur in water and 0.2% (v/v) mild Tween-20 aqueous detergent solutions (Kharat et al. 2024). The germplasm evaluation facilities maintained ambient temperature between 25^0 and 28^0C with 70–80% relative humidity. These plants survived well under a greenhouse available at Department of Molecular Biology And Genetic Engineering, College Of Basic Sciences And Humanities, GB Pant University of Agriculture and Technology, Pantnagar, Uttarakhand (Kharat et al. 2024). While certain strains of *Agrobacterium rhizogene* cause excessive root proliferation in plants when it is found associated with the roots due to virulence. This is popularly known as hairy root disease (HRD) as it was also observed in hydroponically grown tomato, cucumber, and eggplant, resulting in reduced yield (Fortuna et al. 2023) and significant economic losses worldwide (Vargas et al. 2021). However Agrobacterium virulent strains have been observed beneficial in the production of desired metabolites during in vitro cultures in *Picrorhiza kurroa* (Mishra et al. 2011) as they were found to produce signaling molecules triggering metabolism (Kaur et al. 2021).

Bioreactors

Picrorhiza kurroa hairy root cultures using *Agrobacterium rhizogenes* in various nutrient media formulations Gamborg's B5, Murashige and Skoog (MS), WP, and Nitsch & Nitsch (NN) to scale up biomass production in a bioreactor. The scale-up of the selected *Picrorhiza kurroa* hairy root lines in a 5L bench-top fermenter involved evaluating growth performance across different basal media formulations at full and half strength of macronutrients (Verma et al. 2015). Optimization of in vitro culture parameters was found essential for scaling up production of bioactive metabolites. Figure 2.

The yield of root biomass and specific glycosides was highest at pH 6 and lowest at pH 3 while pH 4 and pH 8 showed intermediate levels of production (Verma et al. 2015). Among a number of carbohydrate sources—— namely rhamnose, ribose, mannitol, sorbitol, lactose, galactose, glucose, fructose, market grade sugar and sucrose (control)—— the most suitable carbohydrate source was market grade sugar which exhibited higher yield of total glycoside, kutkoside and picroside I. Full strength B5 medium emerges as the most appropriate choice, but half strength medium produced higher levels of kutkosides and picroside I (Verma et al. 2015). Among leaf, shoot tip and internodal segment, growth potential of resulting hairy roots was relatively higher in shoot tip explants (Mishra et al. 2011). The maximum frequency of root formation was achieved with *A. rhizogenes* strains A4 (Mishra et al. 2011) and the order of frequency was A4, MTCC 15834, PAT 405 and LBA 9402, respectively (Rawat et al. 2016).

Panax ginseng hairy roots report that an appropriate inoculum size could enhance the biomass and active metabolite production, but excess or shortage would decrease growth (Jeong et al. 2004). According to previous studies, maximum active metabolite production was recorded in the sixth to seventh week. The maximum bioactive ingredients (picrotin and picrotoxinin) were found in hairy root culture maintained at 30 ℃. Comparatively lower production of bioactive ingredients was recorded at 25 ℃ and 35 ℃ (Mishra et al. 2011). It indicates that temperature influenced the production of picrotin and picrotoxinin from hairy roots of *Picrorhiza kurroa*.Thiamine HCl greatly persuade the

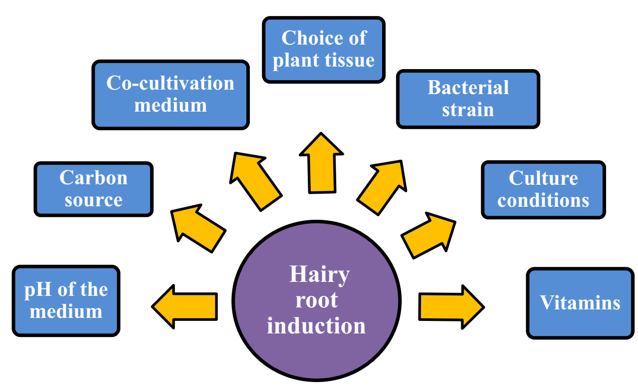

Fig. 2 Parameters affecting Hairy root induction

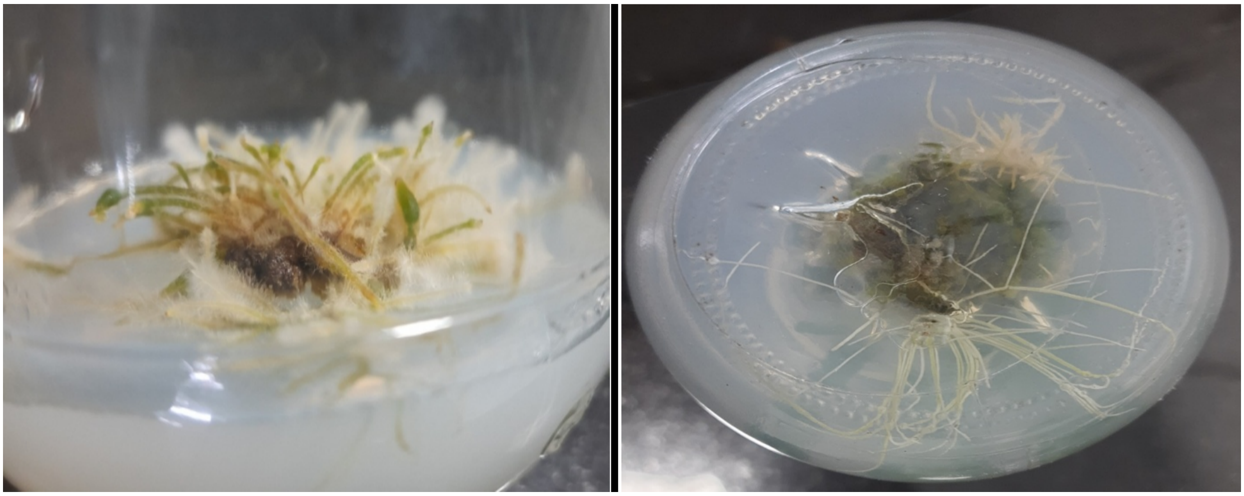

Fig. 3 Hairy root development in *Picrorhiza kurroa* with the help of leaf pieces as explants under inoculation in MS basal medium with specified elicitors

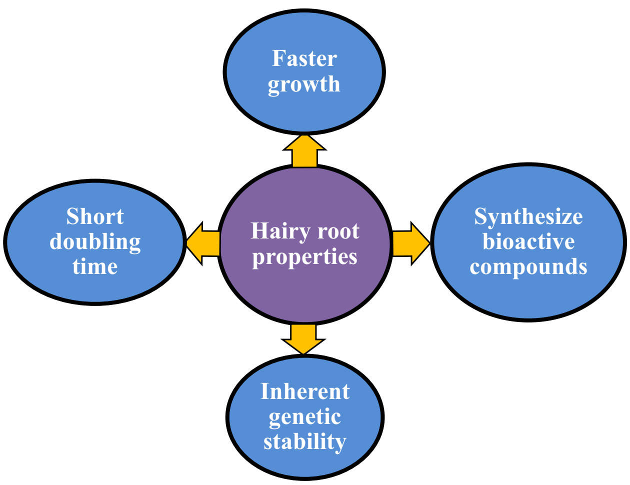

Fig. 4 Properties of hairy root affecting growth and development

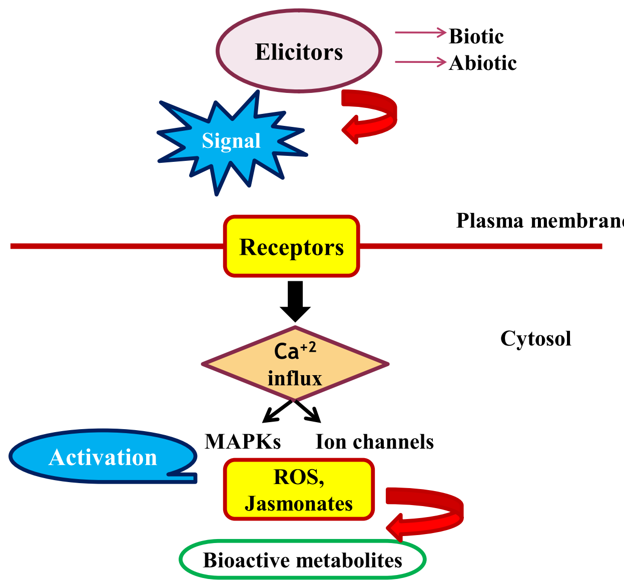

Fig. 5 Representation of Biotic and Abiotic mode of action of elicitors in plant cells

overall active metabolite production in half and full strength of MS medium (Verma et al. 2015; Parkhomenko et al. 2020).

Induction and establishment of hairy root culture

Figures 3 and 4.

Mode of action of elicitors

Elicitor-receptor interactions begin when the elicitor binds to plasma membrane receptors (Kohl *et al*. 2019), triggering calcium ion (Ca^{2+}) efflux from extracellular background and intracellular Ca^{2+} reservoir into the cytoplasm. This process activates signaling pathways, including mitogen-activated protein kinase (MAPKs) and ion channels. These pathways transmit the signal to downstream cascades, inducing the production of reactive oxygen species (ROS) and hormonal signals (jasmonic acid), which leads to bioactive metabolite synthesis (Blume et al. 2000). The interaction of multiple interconnected components involved in signal transduction pathway makes the elicitation process quite complex. This complexity leads to variations in plants responses depending upon the elicitor(s) applied. Figures 5 and 6.

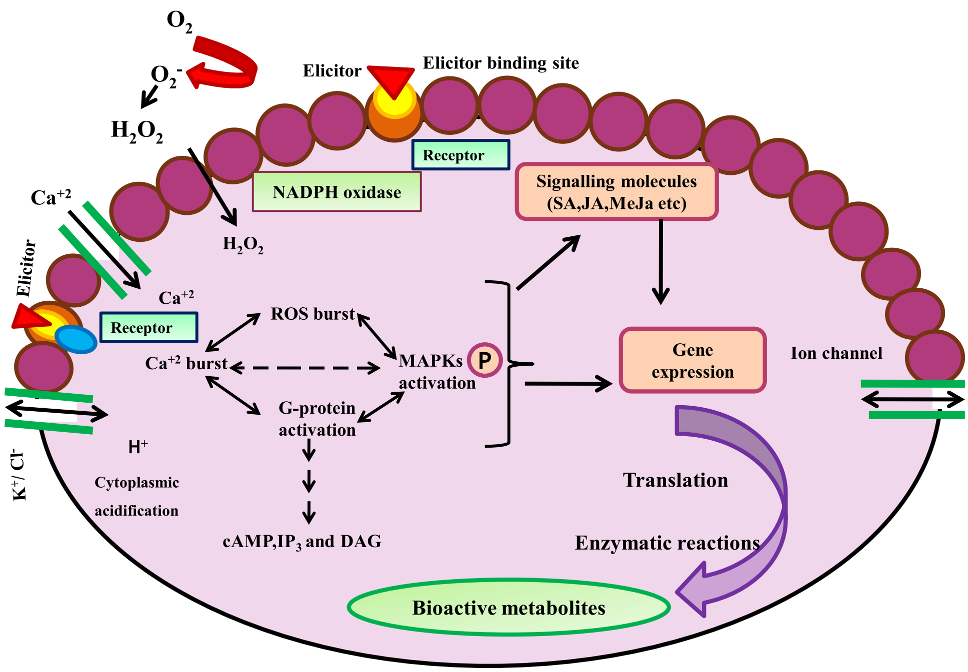

Fig. 6 Diagrammatic representation of cross-talk of elicitors with receptors

Elicitor's nature used for developing morphogenic traits like hairy roots

Panax ginseng hairy roots, when exposed to distinct light spectra, can influence growth and metabolite biosynthesis (Ghimire et al. 2017; Yu et al. 2005). Osmotic agents such as mannitol, cadmium chloride, sorbitol, potassium chloride, sodium chloride, and polyvinylpyrrolidone (PVP) induce osmotic stress in plant cells (Jeong and Park 2006). In *Salvia miltiorrhiza* hairy root cultures, the application of sorbitol resulted in increasing the overall tanshinone content in the roots (Shi et al. 2007). When *Solanum khasianum* hairy roots subjected to salt stress, an enhancement in solasodine content was observed (Srivastava et al. 2016). Metal oxide nanoparticles, including titanium oxide, zinc oxide, iron oxide, and copper oxide, have proven their potential in promoting plant growth and enhancing the production of several metabolites (Kim et al. 2017; Marslin et al. 2017). Nanosilver particles proved to be effective elicitors for *Datura metel* in hairy root cultures, resulting in a significant improvement in both root biomass accumulation and atropine yield (Shakeran et al. 2015). Furthermore, the biomass of *Cucumis anguria* hairy root cultures substantially increases when exposed to AgNPs. Hairy roots elicited by AgNPs exhibited an increase in the total phenolic and flavonoid contents compared to those elicited by Ag + ions (Chung et al. 2018). In *Hyoscyamus reticulatus* L, iron oxide nanoparticles (FeNPs) led to a remarkable approximately five-fold increase in hyoscyamine and scopolamine production at various exposure times (Moharrami et al. 2017). Additionally, the application of copper oxide nanoparticles (CuONPs) as elicitors on hairy roots of *Brassica rapa* spp. pekinensis resulted in a significant elevation of glucosinolate levels. This increase was attributed to the upregulation of MYB factors regulating genes encoding enzymes in the glucosinolate biosynthesis pathway (Chung et al. 2018). Figure 7.

Treatment of *Rauvolfia serpentina* hairy root cultures with mannan and NaCl led to a significant increase in terpenoid alkaloids, specifically ajmaline and ajmalicine compared to untreated cultures (Srivastava et al. 2016). Hairy roots of *Withania somnifera* elicited with salicylic acid exhibited higher yields of withanone, withanolide A, and withaferin (Pandey et al. 2016; Sivanandhan et al. 2013). *Azadirachta indica* hairy root cultures, when elicited with salicylic acid,

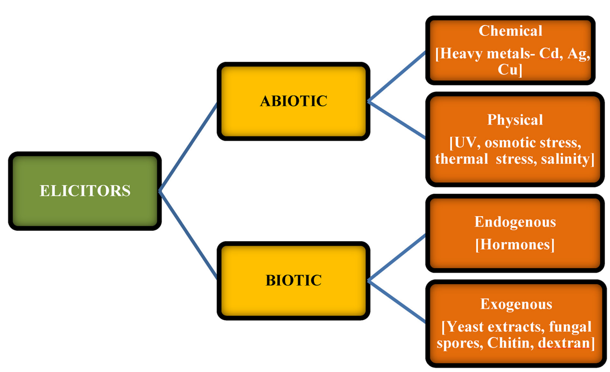

Fig. 7 Classification of elicitors involved in bioactive metabolite production

yielded a maximum amount of azadirachtin compared to the control (Satdive et al. 2007). Salicylic acid has also been found to impact the terpenoid metabolism of various plants, inducing the accumulation of triterpenoids such as glycyrrhizin in licorice and ginsenosides in ginseng (Gwi-Taek et al. 2005; Shabani et al. 2009). Methyl jasmonate (JA) has been identified as a unique elicitor that induces an increase in metabolic flux in Catharanthus roseus hairy root cultures (HRCs), resulting in enhanced specific yields of ajmalicine, lochnericine, serpentine, and horhammericine (Rijhwani and Shanks 1998; Shanks et al. 1998). JA and its methyl esters were recognized for their role in signal transduction pathways and have demonstrated a positive impact on active metabolite biosynthesis in more than 36 plant species (Gundlach et al. 1992). Furthermore, these elicitors have been successfully employed in hairy root cultures of various medicinally important plant species, significantly improving the production of principle metabolites in species such as *Hyoscyamus muticus* (Biondi et al. 2000), *Panax ginseng* (Yu et al. 2000), *Valeriana locusta* (Kittipongpatana et al. 2002) and *Taxus × media var. Hicksii Rehd* (Furmanowa and Syllowska 2000).

Enzymes such as pectinase, cellulose, and several others function as biotic elicitors, capable of triggering the release of endogenous elicitors from plant cell walls. These elicitors are molecular compounds that act as internal signals within plant defense mechanisms and can originate from various microorganisms, including glycoproteins, glucans, various fungal cell wall components, fungal toxins, or fungal culture filtrates (Eilert 1987; Dixit et al. 2020).

Treatment of W. somnifera HRCs with 3% fungus *Piriformospora indica* cell homogenates for 48 h resulted in a significant enhancement in biomass and the production of withanolides, including withaferin A, withanolide A, withanoside V, and withanoside IV (Saxena et al. 2017). Furthermore, several fungal cell wall components and fungal enzymes have demonstrated a slightly positive impact on the production of metabolites in hairy root cultures of various plant species, including *Lippia dulcis* (Sauerwein et al 1991), *Hyoscyamus muticus* (Sevon et al. 1992), *Trigonella foenum-graecum* L (Merkli et al. 1997), *Atropa belladonna* (Lee et al. 1998), Psoralea species (Bourgaud et al. 1999), *Swertia chirata* (Keil et al. 2000), and *Polygonum tinctorium* (Young et al. 2000).

Discussion

Agrobacterium rhizogenes mediated hairy root cultures offer several advantages for producing key bioactive compounds like picroside I, picroside II, kutkoside (Debnath et al. 2020) and picrotin, picrotoxinin (Mishra et al. 2011). The article thoroughly examines how *Agrobacterium rhizogenes* strains, particularly A4, MTCC 15834, PAT 405, and LBA 9402 (Rawat et al. 2016) induce hairy root formation with varying efficacy. These resulting roots exhibit rapid growth, genetic stability, and hormone-independent development while maintaining the biosynthetic capabilities for producing such metabolites. Bioreactor technology is essential for scaling up production to commercial levels (Verma et al. 2015), but further optimization of various parameters for large-scale cultivation of plant cells is needed to ensure consistent metabolite profiles. Hairy root technology offers a promising solution to conservation challenges while meeting pharmaceutical demand. Therefore, the combination of genetic transformation, optimized culture conditions, and strategic elicitation provides a viable alternative.

Conclusion

Picrorhiza kurroa with special reference to hairy root cultures becomes more important owing to in vitro fastest growth compared to other organs. Bioactive metabolites picroside I, picroside II, kutkoside (Debnath et al. 2020) and picrotin, picrotoxinin (Mishra et al. 2011) are also reported in hairy roots in quite good amount even in comparison with in vitro grown cells/ tissues/ organ other than hairy roots. Media (MS) basal components beside, elicitors and other growth regulators are also noticed influencing metabolites during our own study (Bameta 2018). Research has demonstrated that factors such as temperature, osmotic stress, nanoparticles, and other elicitors can further boost metabolite yields. The use of bioreactors further facilitates the scalability of hairy root cultures, making commercial production more viable. In such a manner, in vitro maintained cultures are seemingly viable alternatives alongside traditional cultivation methods. The plant is also being observed more susceptible as a consequence of climate change. Himalayan ecosystem fragility as a result is under various shift mechanism(s) considering bioactive metabolites production. Such changes might further result into dynamic behavior of plant, altering many traits. Strategies for further sustainability of the plant with special reference to active ingredient(s) are needed. Thus, changing behavior of the plant is an observed fact. Through metabolic engineering, elicitation strategies and controlled cultivation, *P.kurroa* hairy root cultures present a sustainable alternative to harvesting from wild habitat. Such studies pave the way to cater to the increasing demand for *Picrorhiza kurroa* in local and global market for the use of various commodities which are ought to essential for the community as such.

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Author contributions Bhawna Pandey generated the idea, drafted the article. A.K. Gaur supervised and edits the manuscript.

Data availability Data available within this article and/or its supplementary material.

Declarations

Conflict of interest The authors declare no conflicts of interest.

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