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Biotechnological approach towards establishment of efficient production alternative for root-derived phytomolecules with optimized yield enhancement parameters from *Agrobacterium* mediated "hairy root" cultures of *Picrorhiza kurroa* Royle ex Benth

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Abstract
The plant *Picrorhiza kurroa* Royle ex. Benth is a fast depleting medicinal value plant. This plant yields a crystalline product called ‘‘kutkin,’’ in its root, which is usually a mixture of two major C9-iridoid glycosides, i.e., picroside-I (6-Otrans-cinnamoyl catalpol) and kutkoside (10-O-vaniloylcatalpol). The increasing market demand of this phytoconstituent has led to threat of extinction of such a valuable endangered medicinal plant species. Attention has already been focused towards alternatives of developing production of root-derived phytomolecules in order to meet the growing demand of pharmaceutical industries. The role of *Agrobacterium rhizogenes* mediated ‘‘hairy root’’ cultures as an efficient production alternative has undeniably proved its effectiveness in the worldwide arena. This article explores and review current technologies available to develop an optimized *in vitro* regeneration protocol for large-scale multiplication and *Agrobacterium*-mediated transformation for induction and establishment of hairy roots of this valuable Indian Himalayan region plant. This plant is consisting of a wide variety of active ingredients and also utilized for establishment and selection of superior individual hairy root clones to enhance the production of root derived phytochemicals from genetically stable *Agrobacterium* mediated hairy root cultures. The outcome of the present report will elucidate approaches for successful induction, growth, maintenance and selection of the rapidly growing hairy root line of *P. kurroa* with *in vitro* production of desired phytomolecule production potential. The present report reviews the superiority of the selected hairy root clone in terms of root biomass and kutkoside/picroside yield over the reported field-grown *P. kurroa* plant will undoubtedly highlight the significance of the biotechnological approach to be applied, which can endow us with a better substitute for the exploitation of this commercially important, medicinal plant species. Due to endangered status of plants it is urgently required to save this medicinal wealth from extinction and exploit different conservational strategies for its better utilization.

Keywords: *Picrorhiza kurroa*, *Agrobacterium*, hairy root cultures, kutkin, transformation, *in vitro* regeneration

1. Introduction
Biotechnology offers avenues for maintenance, genetic improvement and efficient use of endangered plant resources and products [1, 2]. Tissue culture is used for conservation of biological diversity by multiplication of plant species that have extremely small populations, for species with restricted reproductive capabilities and for recovery and reintroduction [3]. World health organization estimates world 80% population relies on traditional medicines [4]. *In vitro* propagation of rare and threatened plants is generally undertaken to enhance the biomass and conserve the germplasm especially when population numbers are low in the wild. *P. kurroa* Royle ex. Benth commonly known as Kutki, a fast depleting medicinal value plant belongs to family Scrophulariaceae. It is endemic and grows in inner ranges of alpine Himalayas, from Kashmir to Sikkim, of Indian state at an altitude of 3,000-5,000 feet above sea level [5, 6]. The roots and rhizomes of 3–4-year-old *P. kurroa* plants yield a crystalline product called ‘‘kutkin,’’ which is usually a mixture of two major C9-iridoid glycosides, i.e., picroside-I (6-Otrans cinnamoylcatalpol) and kutkoside (10-O-vaniloylcatalpol) [7].

Unfortunately, the root of this plant is directly being sold in the local market @ Rs 400-500/kg [8]. The increasing market demand of the plant has led to unscientific, unmanaged and overharvesting of the plant, raising concern amongst many ecologists, scientists and conservationists [9-11]. As a result, the species has been included in the Negative List of Export by the Govt. of India and Appendix II of CITES. Based on the extensive collection for trade and dwindling population in the wild, the plant has also been categorized as Vulnerable in the Red Data Book [12] and as 'Endangered' as per Conservation Assessment and Management Planning (CAMP) workshop [13]. Realizing the threat of extinction of such a valuable endangered medicinal plant species, attention has already been focused towards developing production alternatives of root-derived phytomolecules in order to meet the growing demand of pharmaceutical industries. The stride of hairy root technology from the boundaries of research laboratories to industrial-scale production strategies has magnificently been manifested through the advent of the German company ROOTec (http://www.rootec.com), devoted fully towards up-scaling the hairy root technology as a production alternative at the industrial level for two important phytoconstituents of endangered plant origin. For massive production of secondary metabolite [14] and phytochemicals [15]. Hairy root culture was developed as the innovative path. Thus for developing huge quantity of roots and secondary metabolites in short span of time this technique is most significant for continuous supply of improved value products [16]. The plant developed through *in vitro* regeneration need to be field evaluated and along with the collected field data as complete packages could be ready for commercialization and transfer to the user agencies.

Thus, the present report is focused to explore the technologies to optimize the different parameters to enhance productivity of picroliv as well as some other novel active ingredients from hairy roots of *P. kurroa*. By using different parameters potential in terms of both biomass and secondary metabolites during different growth phases of selected hairy root line of *P. kurroa*. Some available research finding also highlights the significance of optimum conditions for hairy root cultures to investigate its feasibility for the large scale culture in bioreactor and culture conditions on growth and active ingredient (i.e., kutkoside and picroside I) production.

2. Review of Status of Research and Development of Alternative Biotechnological Approaches

(i) International Status

The constituents of *P. kurroa*, identified picroside I, a bitter principle of the subterranean [17], Picrosid-II [18] and a cucurbitacin glycoside [19]. Simons *et al.* [20] reported isolation of five low molecular compounds from a root extract of this plant, which affect the respiratory burst in activated human polymorphonuclear leucocytes and also have immunomodulation activity. Stuppner and Wagner [21, 22] reported new cucurbitacin glycosides, minor iridoid and phenol glycosides of this plant. Minor cucurbitacin glycosides from this plant were discovered and reported by Stuppner *et al.* [23]. Consequently, Stuppner *et al.* [24, 25] reported Cucurbitacins as well as its unusual side-chains forms. Jia *et al.* [26] reported Pikuroside: a novel iridoid of this plant.

Ex situ conservation provides a better degree of protection to germplasm compared to *in situ* conservation. Therefore, concerted efforts of both *in situ* and *ex situ* conservations are needed and should not be viewed as alternatives [27]. *Exsitu* conservation includes germplasm banks, common garden archives, seed banks, DNA banks and techniques involving tissue culture, cryopreservation; incorporation of disease, pest and stress tolerance traits through genetic transformation and ecological restoration of rare plant species and their populations. *Ex situ* conservation has gained international recognition with its inclusion in Article 9 of the Convention on Biological Diversity [28].

Laurie *et al.* [19] isolated and identified a novel cucurbitacin glycoside from roots extracts of *P. kurroa*. Stuppner and Wagner [24, 25] has been isolated three glycosides, out of which one was novel iridoid compound and two phenol glucosides from the roots of *P. kurrooa*. In continuation the same group in 1998, isolated seven cucurbitacin glycosides from the roots of *P. kurrooa* and structurally identified by NMR and mass spectroscopy. Stuppner *et al.* [24] identified and isolated the seven new cucurbitacins from the roots of *P. kurrooa* and were determined their structure on the basis of UV, 1H, 13C NMR, 2D 1H,1H-13C NMR and mass spectroscopy. From the roots of *P. kurroa* six new cucurbitacin glycosides were isolated and their structures were established by usual spectroscopic and two-dimensional (2D) NMR techniques [25]. Jia *et al.* [26] isolated a novel iridoid Pikuroside together with three known iridoids from *P. kurroa*. Zhang *et al.* [29-30] identified novel lipid peroxidation enzyme cyclooxygenase and reported its activity as inhibitory tannins and triterpenes.

Picroliv, a *P. kurroa* derivative, on experimental model of UC in mice has been validated to have antioxidant and anti-inflammatory effect. Colitis induced by dextran sulfate sodium (DSS) in mice model and myeloperoxidase (MPO) activity, SOD, MDA concentrations were measured by enzyme-linked immunosorbent assay (ELISA) while the expression of cytokine mRNAs was studied by real time-quantitative polymerase chain reaction and also ELISA [31]. Extraction and purification of bioactive compounds, picroside-I, II and III, in crude herbal extracts from *P. scrophularia* flora using pipette tip solid-phase extraction (PT-SPE) technique has been done using a pipette tip packed with C18. This method proved as fast, convenient, cost-effective and best for analysis of herbal medicine. Thus this report gives good results for analysis of *Picrorhiza* as well as other herbal plants [32].

(ii) National status

Indian pharmacopoeia has listed *P. kurroa* as an official drug (The pharmacopoeia of India, 1970) Kutki has been used in the indigenous system of medicine since a long time, the well-known drug is spoken 'Dharvantarigrasta'. Few reports are available on micropropagation aspect of this plant through clonal multiplication. Its urgent need to exploit this endemic and endangered plant to develop large scale multiplication using various explants as well as to conserve this plant. Along with this, the plant has wide variety of active compounds. Generally, through interventions based on biotechnology commercialization of plant compounds can be carried out without harming their natural territory. Concerning, *P. kurroa,* studies focused on tissue culture and secondary metabolite production utilizing hairy root cultures have been reported previously, out of which few have been discussed here, yet there is no report on bioactive molecules production through tissue culture raised plants and their genetic stature.

Lal *et al.* [33] reported clonal propagation of *P. kurroa royle* ex benth. using shoot tip culture. Vitrification of shoots in cytokinin supplemented medium of *P. kurroa* has been reported earlier, which is showed beneficial or adverse effects of different cytokinins at varying concentrations [34]. Nanda and Ahuja [35] also employed shoot tips for multiplication of this plant for multiplication. Many attempts have been made to

regenerate *P. kurroa* using *in vitro* techniques, success is quite limited [36-37]. Chandra *et al.* [38-39] reported micropropagation of this plant using node and shoot tip explants as well as conservation of this endangered alpine herb of Himalayan region with high commercial value. Sood and Chauhan [40-41] subsequently reported development of a low cost micropropagation technology and further high frequency callus induction and plantlet regeneration of this plant. Jan *et al.* [42] developed improved micropropagation protocol of this plant using auxin treatments. *In vitro* shoot multiplication through sprouting of axillary buds using nodal and leaf explants reported by Sharma *et al.* [43]. Patial *et al.* [44] developed propagation methods and *ex situ* conservation of this plant using leaves from aseptic shoot cultures, raised from *ex vitro* leaves. Gupta *et al.* [45] reported micropropagation through callus mediated organogenesis and try to estimate the content of P-I in different *in vitro* grown developmental stage. Further, HPLC analysis of various *in vitro* cultured morphogenetic stages exhibited that the leaf explants have higher P-I content as compared to the root explants.

There are a few reports of *Agrobacterium rhizogenes*-mediated production of transformed hairy root cultures in *P. kurroa* [46-47], there are very few reports available for the *Agrobacterium tumefaciens*-mediated transformation of this highly valuable medicinal herb. Bhat *et al.* [48] first time reported efficient plant regeneration via direct organogenesis using leaf explants and *Agrobacterium tumefaciens*-mediated genetic transformation. Significant success has been reported in enhancing the production of key secondary metabolites in plants by the genetic manipulation of biosynthetic pathways using genetic transformation [49-52]. Guillon *et al.* [53] exploited the potential of hairy roots and *Agrobacterium rhizogenes* induced hairy root cultures were utilized as advantageous resource of useful plant based compounds. Hairy root culture has transformed the function of *in vitro* culture for the secondary metabolite production [54]. A foremost feature of hairy root is their general ability to produce the preferred phytochemicals along with growth. In this respect Bourgaud *et al.* [55] reported unlike the production being repressed during the growth phase of dedifferentiated cell culture, it is possible to get constant and standardized production of the desired phytochemicals from growing hairy roots. The biosynthetic potential of the transformed roots is genetically controlled but it has frequently been observed that such transformed roots are sensitive to medium composition with respect to both biomass yield and secondary metabolite productivity [56-57]. Several groups have shown that besides selection of superior hairy root clones with higher than average productivity; media optimization could enhance the biomass as well as secondary metabolite yield [57]. Kumar *et al.* [58] reported that its roots possess much bitterness and are used medicinally by the natives. Sood and Chauhan [59] reported the biosynthesis and accumulation of Picroside-I, a medicinal compound in leaf discs, stem and root segment cultures of *P. kurroa* Royle ex Benth. Only in the specialized cells of *P. kurroa* the biosynthesis and accumulation of picrosides occur, mainly in *in vitro* cultures whereas, no picrosides were detected in the callus cells of this plant, however, P-I was detected in the cultures differentiating into shoots [60]. Pandit *et al.* [61] reported differential biosynthesis, which includes variable amounts of P-I and P-II growth and developmental stages along with accumulation of picrosides, implies importance of selection of plant material (rhizomes and roots) in *P. kurroa*. Yield enhancement strategies for the production of picroliv from hairy root culture of *P. kurroa* Royle ex Benth reported by Verma *et al.* [62]. They have investigated the effects of various nutrient medium formulations viz B5, MS, WP and NN, and sucrose concentrations (1-8%) on the biomass and glycoside production of selected clone (14-P) of *P. kurroa* hairy root.

P. kurroa has many medicinal benefits such as immunomodulatory, anti-allergic, anti-anaphylactic, hepatoprotective and anti-neoplastic activities [63-65]. The flavonoid apocynin is one of the active metabolites of *P. kurroa* and has been reported to attenuate Parkinson's, hypoxia and ischemia-reperfusion by its inhibitory action on NADH oxidase; expressed during oxidative stress [66-68]. Along with these, *P. kurroa* also exhibited potential anti-diabetic activity [69].

3. Biotechnological methods to be applied

Runners of *P. kurroa* should be collected from appropriate region. Some reports indicate that there is considerable degree of variation in amount of active ingredients level among populations of rhizomes of *P. kurroa* Royle ex Benth growing at different altitudes of Himalayas [70]. Literature search and required test should be done to collect *P. kurroa* with high amount of active ingredients and brought to the laboratory to develop *in vitro* cultures. *In vitro* grown plants of *P. kurroa* are taken as the source of explant. These *in vitro* grown plants should be maintained at 25 °C under a 16-h photoperiod regime of fluorescent light (30 mmol $m^{-2}s^{-1}$). Leaf, shoot tips and internodal segments excised from the shoots of *in vitro* grown plants can be used as explants for the present study. Standard plant tissue culture media like Murashige and Skoog (MS) and Gamborg (B5), etc. may be used in the study. These media may be supplemented with different combinations and concentrations of plant growth regulators like BA, Kinetin, IBA, IAA, NAA, 2,4-D, and TDZ, etc. according to need of experimentation to achieve axillary shoot proliferation, adventitious shoot bud regeneration and somatic embryogenesis to produce large number of genotypically identical plants in a relatively short period of time. For Induction and establishment of hairy roots *P. kurroa* plants (8–10 weeks) should be maintained under *in-vitro* conditions on semisolid MS (Murashige and Skoog) medium to be used as explant source. Different strains of *A. rhizogenes*can should be subcultured on Luria–Bertani (LB) medium. Prior to infection, the bacteria should be grown for 48 h in 50 ml of liquid LB medium [71] at 28 ± 2 °C on a rotary shaker at 90 rpm in the dark. Leaf, shoot tips and internodal segments of the plant are used as explants for hairy root induction. The young leaves and stem segments should be inoculated/wounded by pricking with a sterile needles dipped in bacterial suspension with suspension culture of *A. rhizogenes* strains (e.g. A4 and PAT 405) grown in liquid YMB [71] medium (OD600 = 0.9–1.0) at various time intervals (1/2, 1, 12, 24 and 48 h). After 48 h of co-cultivation with the individual bacterial strain, the explants should be transferred onto the same respective medium containing the antibiotic carbenicillin (250 mg/l) or 1.0 gL^{-1} of cephalexin under dark conditions. Similar types of explants, pricked with a sterile needle devoid of the bacterial suspension should be cultured under uniform conditions as controls. After 14–21 days of culture number of explants producing hairy roots are obtained. The hairy roots which emerged from the infected sites should be maintained at standard culture conditions of 25 ± 2 °C temperature and 55–60% relative humidity in half-strength hormone-free liquid MS medium with 3% sucrose on a rotary shaker at 90 rpm for their further proliferation. Once established, the individual hairy root clones are transferred to liquid B5 medium with the same

concentration of antibiotic and incubated on a rotary shaker in the dark at 25 ± 1 °C under constant agitation (80-90 rpm). The crushed hairy root extracts should be streaked on semisolid YMB medium to check for the presence of *A. rhizogenes*. These hairy root lines should be repeatedly subcultured in half-strength semisolid MS medium after every 3 weeks to reduce the concentration of antibiotic. Roots excised from *in vitro* grown complete plantlets of *P. kurroa* should be cultured under identical conditions in liquid B5 medium supplemented with appropriate concentration of plant growth regulators to serve as control roots. Hardening of the rooted plantlets is attempted by gradually transferring them from high humidity-low light to natural light-humidity conditions and the plants can be then transferred to soil. After transferring the plants to field, field evaluation for different characteristics of plants can be performed.

Growth kinetic studies can be performed by evaluating independently generated hairy root clones based on total root elongation (cm), lateral branching per centimeter of primary roots and fresh weight (FW) increment after 15 days of incubation in full- and half-strength liquid B5 medium containing sucrose. The different growth media formulations, for example MS (Murashige and skoog), Gamborg's B5, NN (Nistch & Nistch) at both full and half strengths may be used for optimizing the yield potential of the selected hairy root clone. Best calculated inoculums density of 1.8 g fresh weight of freshly grown root should be inoculated in triplicate to 50 ml of hormone-free full or half concentrations of the basal media. The growth analyses of the roots should be done at 20-day interval up to 60 days. Based on the apparent growth behaviors with respect to these specific parameters, best individual root clones should be selected for further studies. All selected hairy root clones and the control, non-transformed roots can be subjected to growth kinetic analysis for growth kinetic studies, 100 mg of actively growing hairy roots from 15 days old cultures should be transferred to 250-ml Erlenmeyer flasks containing 50 ml of optimized growth media with sucrose, and their growth performances may be determined following the method described by Verma *et al.* [72]. The selected superior hairy root clone can be cultured in optimized growth media for scale up studies.

Characterization of hairy roots may be performed to prove the transformed nature of the selected hairy root clones. Opines may be extracted and detected by electrophoresis according to the procedure of Morgan *et al.* [73] in parallel to extracts from non-transformed *in vitro*-grown control roots. Their transformed nature can be further ascertained through PCR analysis according to the procedure described by Rahman *et al.* [74] using TL specific primer. For the confirmation of transgenic nature of hairy roots, the DNA of hairy roots and control plant can be isolated from 50 mg fresh tissue following the protocol of Doyle and Doyle [75] with required modifications. Ten milliliters of preheated extraction buffer [2% CTAB (w/v), 0.2% β mercaptoethanol (v/v), 100 mM Tris–HCl (pH 8.0), 20 mM EDTA, and 1.4 mM NaCl] may be added to the powdered material. The DNA pellet can be resuspended in 200–300 mL TE (1 mM) and quantification can be performed by visualizing under UV light, after electrophoresis on 0.8% agarose gel stained with ethidium bromide. To prove the transformed nature of hairy roots, polymerase chain reaction (PCR) analysis may be done. Primers for detecting rolB gene (5'-CTTATGACAAACTCATAGATAAAGGTT-3' and 5'-TCGTAACTATCCAACTCACATCAC-3') can be used in amplification process. PCRs can be carried out in a final volume of 25 µl containing 20 ng template DNA, 200 µM each deoxynucleotide triphosphate, 20 ng of primers, 1.5 mM $MgCl_2$, 10 mM Tris–HCl, 50 mM KCl, 0.1% Triton X-100 and 0.5 U Taq DNA polymerase. Amplification may be achieved in a Thermocycler programmed for a preliminary 5 min denaturation step at 94 ^{0}C, followed by 40 cycles of denaturation at 94 $^\circ$C for 30 s, annealing at 52 °C for 1 min and extension at 72 °C for 1 min, finally at 72 °C for 10 min. Alternatively, southern blot analysis may be conducted according to Choi *et al.* [76]. Total genomic DNA should be isolated from leaves of control plants (non-transformed plant) and plants derived from hairy roots. Total DNA from each plant should be digested with Sal I, electrophoresed on a 1% agarose gel and transferred to a Hybond N^+ membrane. In the same manner, total DNA from *A. rhizogenes* strain should be transferred to the membrane. A 3.2 kb DNA fragment containing the TL-DNA sequence can be amplified with the primers 5'-ATGGCATTCAATAACGACCGTAC-3' and 5'-CATCGCACTGGCTATGTCG-3' and used as a probe. Probe DNA to be labeled. Prehybridization and hybridization can be performed in saline sodium citrate (SSC), Denhardt's solution, and 0.5% (w/v) sodium dodecyl sulfate (SDS) at 65 °C overnight. The membranes should be finally washed with SSC and 0.1% (w/v) SDS at 65 °C for 30 min and picture should be taken.

Study of effect of different elicitors for example yeast extract, methyl jasmonate etc. on the production of phytochemicals from selected hairy root clones can be studied with the aim to enhance the production of valuable phytochemicals. Further chemical analysis can be performed to determine the time course production of the desired secondary metabolites, i.e., kutkoside and picroside I, by subjecting the dried root samples to a chemical extraction process. The extraction of glycosides and HPLC analysis for kutkoside and picroside I can be carried out according to the procedure reported by Gupta [77] with required modifications. For extraction of active ingredients hairy roots induced by *A. rhizogenes* strains should be harvested from 8-week-old culture. The fresh weight and dry weight after lyophilization can be determined. The powdered samples (0.5 g DW) can be extracted with 100 ml of 70% ethanol in a soxhlet apparatus, and then dried crude extract should be dissolved in 10 ml of water:methanol:isopropanol:acetonitrile (60:30:5:5) and quantification by HPLC may be done using standards [78]. A standard curve can be prepared by 10, 20, 30, 40 and 50 mg/l solutions of picrotin and picrotoxin standard (Sigma Chemical Company, St. Louis, USA). The compounds will be identified on the basis of their retention time and comparison of UV spectra with the authentic standards. Picroside-II content can be estimated using high performance liquid chromatography (HPLC) analysis method developed by Sood and Chauhan [59]. P-II content in 15 °C roots has already been experimentally proven to be 0mg/g fresh weight [61]. For quantification of P-II in the stolon samples, they should be dissolved in methanol after being grounded into a fine powder using liquid nitrogen. The filtered extract should be then diluted 10 times and analyzed using reverse phase (HPLC) through C18 (5 m) 4.6 × 250mm Waters Symmetry Column using PDA detectors. Two solvent systems may be used for running the test samples, that is, solvent A (0.05% trifluoroacetic acid) and solvent B (1:1 methanol/acetonitrile mixture. Solvents A and B be used in the ratio 70:30 (v/v). The column can be eluted in isocratic mode with flow rate of 1.0 mL/min. P-II can be detected at 270 nm. The cycle time of analysis should be 30 minutes at 30 °C. The compounds will be identified on the basis of retention time and

comparison of UV spectra with the authentic standard. The quantification should be repeated thrice for each sample, and the data can be subjected to appropriate statistical analysis for a dependable interpretation of the results.

For protein extraction, the samples should be excised and washed with sterile water. These washed and dried samples should be frozen in liquid nitrogen and grounded in a precooled pestle and mortar to obtain a fine powder. This fine powder should be then suspended in 10% (w/v) trichloroacetic acid (TCA) in 100% (v/v) acetone containing 0.07% (w/v) dithiothreitol (DTT). For complete precipitation, samples should be incubated overnight at −20 °C, followed by centrifugation at 15,557×g for 45 minutes. The pellets should be resuspended in 100% (v/v) acetone containing 0.07% DTT for 1 hour, followed by centrifugation at 15,557 ×g for 45 minutes. This step should be repeated thrice to completely remove any residual TCA. The pellet should be then air dried to remove acetone and resuspended in lysis buffer containing 7M urea, 2M thiourea, 2% (w/v) 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 1% (w/v) DTT, 2% biolyte pH3–10, and protease inhibitor cocktail. The samples should be then sonicated in a water bath maintained at 20 °C for 30 minutes followed by gentle stirring at room temperature for 4 hr. The samples should be centrifuged at 15,557 ×g for 45 minutes and supernatant obtained should be transferred to a new tube for further centrifugation at 15,557 ×g for 45 minutes to remove any residual precipitate. The clear supernatant obtained are then divided into aliquots and stored at −80 °C. Protein concentration may be estimated by Bradford method. For SDS-PAGE analysis, the required amount of the samples may be precipitated by adding 100% (v/v) acetone containing 0.07% DTT in 1:4 ratio. The samples should be kept at −20 °C for at least 2 hours, followed by centrifugation at 20,000×g for 15 minutes. The pellet obtained should be air dried at room temperature and resuspended in 2x Laemmli buffer containing 0.5mM TrisHCl (pH 6.8), 25% glycerol, 1% bromophenol blue, and 10% SDS. The samples may be loaded onto a discontinuous gel system containing 12% resolving gel and 5% stacking gel. Separation may be carried out using electrophoresis setup at 16mA for first 30 minutes, followed by 24mA till dye front reaches bottom of the gel. The gels can be visualized by silver staining. SDS-PAGE gel separations should be repeated for 3 times for confirmation. The SDS-PAGE gels can be scanned using densitometer. All image analysis and densitometry studies can be performed using applicable software. MALDI-TOF/TOF MS Analysis may also be performed as per requirement.

Though transformation of a number of agriculturally important plant species has been reported, such efforts on medicinally important plants have been very few [79-80]. In several cases, plants with highly expressed secondary pathways and a well-defined regeneration system have been reported as recalcitrant to regeneration after genetic transformation, as in *Plumbago zeylanica* [81] and *Euphorbia nivulia* [82]. Wild type nopaline strain of *A. tumefaciens* has been used in genetic transformation of *Mentha citrate* [83] to produce transformed shoot cultures, and *Coleus forskohlii* [84] to develop cell suspension cultures. In *Mentha piperita* L. [85-86], *Artemisia annua* [87], *Scrophularia buergeriana* Miq. [88], *Digitalis minor* L. [89], *Echinacea purpurea* [90] and *Ruta graveolens* [91], gus A reporter gene and npt II selection marker have been used for developing transgenic plants by using leaves/hypocotyl as explants. The utility of *A. tumefaciens* and *A. rhizogenes* in genetic transformation of medicinal and aromatic plants [79, 92] has been reported. Thus the present biotechnological approach can be implemented to optimize the different parameters to enhance productivity of picroliv as well as some other novel active gradients from hairy roots of *P. kurroa*. By using different parameters potential in terms of both biomass and secondary metabolites during different growth phases of selected hairy root line of *P. kurroa*.

4. Conclusion

Due to overexploitation of useful Himalayan medicinal plants, many of the medicinal plants have reached to the verge of extinction. The outcome of the present report elucidates successful method for induction, growth, maintenance and selection of the rapidly growing hairy root line of *P. kurroa* with *in vitro* desired phytomolecule production potential and also review the available reports. The superiority of the selected hairy root clone in terms of root biomass and kutkoside/picroside yield over the reported field-grown *P. kurroa* plant will undoubtedly highlight 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. The prime importance of *in vitro* propagation of rare, critically endangered, endangered and vulnerable medicinal plants of Indian Himalayan region would be to generate a large number of planting materials from a single explant without destroying the mother plant and subsequently their restoration in the natural habitat, thus conserving the biodiversity. The significance of an efficient *in vitro* protocol would be to obtain maximum number of plantlets in minimum period with proper rooting along with acclimatization in the field. Their germplasm could be conserved using slow-growth conservation studies. This report opens possibilities for bioreactor-based production of pharmaceutically important metabolites like picrotin and picrotoxinin without threatening the biodiversity. The developed regeneration systems should be field-tested and the field data should be collected so that the complete technology packages could be ready for commercialization and transfer to the user agencies.

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