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Biotechnological Approach for the Conservation of *Picrorhiza scrophulariiflora* Pennell

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Abstract—*Out of 814 threatened plants of India, Picrorhiza scrophulariiflora Pennell (Scrophulariaceae) is one such threatened species of Indian Himalayan region, having medicinal properties. Even though its existence is in threat due to anthropogenic as well as some other ecological factors, yet there is no any report on proper conservation measures. To check the exact current population status of both the species, the selected populations were analyzed using vertical belt transact model, which revealed that the P. scrophulariiflora is vulnerable in Sikkim Himalayas. Therefore, in order to exploit this high valued species for mass cultivation, simple and highly reproducible protocols for micropropagation, somatic embryos, direct shoot regeneration from callus and production of synthetic seed have been developed after selecting elite line and for this purpose picroside I and picroside II were used as marker molecules. The HPLC analysis revealed that the population of P. scrophulariiflora from Ha, Bhutan contains highest total picroside (i.e., picroside I and picroside II) with a total of 7.33% (picroside I= 2.21 and picroside II= 5.12% respectively). Therefore, the population of Ha, Bhutan was considered as elite line. The morphological and biochemical characters of the studied populations varied exclusively in terms of soil characters as well altitudinal gradients; therefore, attempt has also been made to correlate those soil and altitudinal parameters with the biomass of the species. To check whether these variations in active biomolecules content and biomass of this medicinal plant is due to those environmental factors or due to genetic factors, genetic diversity studies were undertaken using RAPD markers. The dendrogram clustered the individual of P. scrophulariiflora into 4 sub-groups according to the geographical regions indicating the variations in total picroside content may be due to genetic factors rather than the altitude or other environmental factors.*

1. INTRODUCTION

Picrorhiza scrophulariiflora Pennell (Scrophulariaceae) is an endangered medicinal plant with a restricted habitat of sub-alpine and alpine vegetation of eastern Himalayas (Bantawa *et al.,* 2009). The rareness is due to overexploitation for medicinal purposes along with unorganized as well as unscientific collection practices (Manandhar, 1999), the population of this taxon has been dwindling in the wild to such a critical level that a ban on its collection from the wild habitat has been recommended in India (Nayer and Shastry, 1990).

The genus *Picrorhiza* is well characterized chemically for its two species namely, *P. kurroa* and *P. scrophulariiflora.* Although the species *P. kurroa* and *P. scrophulariiflora* are rich source of irridoid glycosides such as picroside-I, II, III and kutkoside as major bioactive compounds, *P. scrophulariiflora* contains an additional phenylethanoids glycosides and plantamajoside which are absent in *P. kurroa* (Li *et al*., 1998). Further, Bantawa *et al.,* (2010) reported that *P. scrophulariiflora* contain significantly higher amount of picroside I and picroside II than in *P. kurroa.* Thus, *P. scrophulariiflora* is not only the substitute but also chemically superior than *P. kurroa* (Smit *et al*., 2000).

The rhizomes of *P. scrophulariiflora* are used for traditional medicines to treat several ailments. The rhizome of *P. scrophulariiflora* are used in Tibetan and Chinese traditional medicine to treat various ailments such as liver disorders, fever, asthma, jaundice, and have pharmaceutical values for hepatoprotective, immunomodular and antiasthmatic activities (Ghisalberti, 1998; Smit *et al*., 2000). In Nepal, rhizome of this species is also used as a cathartic, in case of dyspepsia, as a purgative, as well as in the treatment of scorpion bites (Anon. 1993).

Several reports indicate the need for its conservation, sustainable utilization and cultivation (Olsen, 1998; Manandhar, Bantawa *et al.,* 2009, 2010). Therefore, in order to utilize the elite germplasm of *P. scrophulariiflora* in sustainable manner and for conservation measure, some works have been done in resent past which are reviewed below.

Population status

On the basis of vertical belt transact (Michael, 1990) Bantawa *et al*., (2009) reported that the current status of *P. scrophulariiflora* in Sikkim Himalayas is vulnerable (Table 1) and not endangered as indicated earlier by Nayer and Sastry (1990). This vulnerable status was based on two very important parameters: (i) Extent of occurrence (EOO) and (ii) Mature population estimation (MPE), which are among the many parameters suggested by IUCN Red List Category (2004).

Table 1: Determination of species diversity per unit area and threat status of *P. scrophulariiflora* in Sikkim Himalayas.

Code*	No. of species/m2	Mature population estimated	EOO	Status IUCN (2004)
Thangu I	24	2480	8	Vu^{1-2}
Thangu II	17	890	4	En^{1-2}
Thangu III	22	2291	8	Vu^{1-2}
Chopta I	20	2065	6	Vu^{1} En^{2}
Chopta II	25	2100	8	Vu^{1-2}
Chopta III	16	2354	5	En^{1-2}
Ja Chu I	17	1975	4	En^{1-2}
Ja Chu II	14	2662	5	En^{1} Vu^{2}
Ja Chu III	15	1725	5	En^{1-2}
Kupup I	18	1812	8	Vu^{1-2}
Kupup II	16	1870	8	Vu^{1-2}
Kupup III	19	2437	8	Vu^{1-20}
Gnathang I	23	5221	8	Vu^{1-2}
Gnathang II	20	2711	5	En^{1} $Vu2$
Gnathang III	15	1613	5	En^{1-2}
EOO= Extent of Occurrence, [1]= on the basis of EOO, [2]= on the basis of Population estimation				

Source: Bantawa *et al.,* 2009.

2. PHYTOSOCIOLOGY

Phytosociological studies of this species revealed that *P. scrophulariiflora* prefers to grow in rock crevices, rocky terrain with moist soil. This species is habitat specific and it has very limited ecological amplitude with sparse distribution in sub-alpine zone of North and East Sikkim. Furthermore, West and South-West facing slopes were the most preferred habitat of this species. The most dominant associates of this species were *Rhododendron anthopogan* and *R. setosum* and one of the most important observations was that *P. scrophulariiflora* invariably found to be grown under the canopy of *R. anthopogan.*

3. MEDICINAL PROPERTY

Picrorhiza has long been used in the preparation of a large number of medicinal compounds. Rhizomes of *Picrorhiza* have been used in many Ayurvedic preparations. Of a total of investigation of 444 classical preparations so far, 37 were found to contain rhizome powder of *Picrorhiza* (Bantawa *et al*., 2011a). Most of these preparations are generally used in the treatment of fever, liver disease or skin disease, however, the contents of *Picrorhiza* in these are low. One of the best known Ayurvedic preparations is Arogyavardhini, containing 50 % *Picrorhiza*, used for treatment of lingering fever, obesity, diabetes, skin disease, and liver disease. Several folk medicines, used among tribal and rural communities in India, are also known to contain *Picrorhiza* rhizome powder. In Nepal, *Picrorhiza* is routinely used in preparations prescribed for liver diseases (Anon 1993).

The dried rhizome is reported to contribute to alleviating fever, malnutrition caused by digestive problems, jaundice, diarrhoea, and dysentery. The rhizome of this species is also used as an adulterant of, or as a substitute for, *Gentiana kurroa*.

Phytochemistry

Most reports on biological activities and constituents of *Picrorhiza* deal with *P. kurroa*, while *P. scrophulariiflora* is rarely mentioned. This is due to the fact that both species share almost similar chemical composition, and till now, the only known difference is that *P. scrophulariiflora* contains additional compounds such as phenylethanoids, glycosides, and plantamajoside, which are all absent in *P. kurroa* (Li *et al*., 1998; Smit 2000). Rhizomes of *P. scrophulariiflora* are widely used in pharmaceuticals (Olsen 1998) due to presence of active constituents such as picroside III (Weinges and Kunstler 1977), picroside, kutkoside (Aswal *et al*., 1984), picroside V (Simons *et al*., 1989), veronicoside (Stuppner and Wagner 1989), picroside II (Wang *et al*., 1993), D-mannitol, vanillic acid (Yang 1996), phenylethanoid glycosides, along with iridoid 8 glucoside (Li *et al*., 1998) and pikuroside (Jia *et al*., 1999). Recently, Bantawa *et al*., (2010) found that the contents of picroside I and picroside II are significantly higher in *P. scrophulariiflora* than in *P. kurroa*.

Selection of elite clones

The main iridoid glycoside reported from *Picrorhiza* is 'kutkin', for which it is valued for. It is a mixture of picroside I and picroside II, which is responsible for the hepatoprotective activity (Shukla *et al*., 1991). Among the many pharmaceutically active molecules, picroside I and picroside II are the most important glycosides. Based on these facts Bantawa *et al*., (2010) analyzed these two bioactive molecules using High Performance Liquid Chromatography (HPLC) from different populations of Eastern Himalayas including Eastern Nepal, Sikkim (India) and Ha (Bhutan) (Table 2). Among these studied populations significantly higher percentage of picroside I and picroside II content were found to be present in the rhizomes collected from Ha, Bhutan. Furthermore, the population of Ha, Bhutan showed gross morphological superiority over other studied populations. Therefore, the population of Bhutan was considered to be as elite line.

Table 2: Morphological descriptions and Picroside I and II contents in different *Picrorhiza* rhizomes.

Picrorhiza spp.	Altitude	Rhizome			Picroside I (%)	Picroside II (%)	Total (%)
		Diameter (cm)	Length (cm)	Dry weight (g/rhizome)			
P. Kurroa	Palampur, Himachal Pradesh, 3,000 m	0.45 ± 0.4d	7 ± 0.23 c	1.45 ± 0.16c	0.55 ± 0.23d	1.34 ± 0.24 d	1.89 ± 0.47 c
P. scrophulariiflora	Thangu, North Sikkim (4,000 m)	0.67 ± 0.05 b	9.74 ± 0.52 b	1.56 ± 0.14 b	0.95 ± 0.05 c	5.40 ± 0.56 a	6.35 ± 0.61 b
-do-	Kupup, East Sikkim (4,200 m)	0.64 ± 0.05 c	6.43 ± 0.41 d	1.41 ± 0.05 d	2.99 ± 0.12 a	4.17 ± 1.02 c	7.16 ± 1.14 a
-do-	Ha, Bhutan (4,200 m)	0.7 ± 0.12 a	11.89 ± 0.4 a	2.12 ± 0.31 a	2.21 ± 0.56 b	7.33 ± 0.68 a	

* Data (mean ± SE) pooled from three independent experiments; mean followed by same latter does not differ significantly according to Duncan Multiple Range Test. ($p \leq 0.05$)

Source: Bantawa *et al*.,, 2009.

4. SHOOT PROLIFERATION FOR CLONAL MASS PROPAGATION

In vitro shoot proliferation offers a useful tool for conservation of germplasm and mass propagation of threatened plant species (Coste *et al*., 2012). Further, *in vitro* clonal propagation of medicinal plants allow for large-scale production of therapeutically high valued taxa for commercialization and sustainable utilization in the industrial sector (Xu *et al*., 2012). *De novo* regeneration has also been reported for a growing list of medicinal and aromatic plants (Pant *et al*., 2010).

Although several parts of the plant have been used as explants, nodal segments of mature plants have been widely used (Bantawa *et al*., 2012). Recently, shoot-tips and nodal explants for proliferation of *P. scrophulariiflora* were successfully used (Bantawa *et al*., 2009b; Bantawa *et al*., 2010). While Murashige and Skoog (1962) (MS) is the main basal media, yet different growth regulators, such as 6-benzyladenine (BA) thidiazuron (TDZ), and kinetin (Kin), either alone or in combination with indole-3-acetic acid (IAA), a-napthaleneacetic acid (NAA), or indole-3-butyric acid (IBA) have been used mostly for inducing bud-break and axillary shoot proliferation, as well as for subsequent shoot elongation. For shoot proliferation of *P. scrophulariiflora,* lower concentrations of Kin (0.5 mg/l) alone were preferable. Addition of IAA or NAA along with Kin did not improve the frequency of shoot proliferation, but instead increased vitrification and induced callusing of shoot cultures (Bantawa *et al*., 2009b).

5. ROOTING AND ACCLIMATIZATION OF SHOOTS

For *P. scrophulariiflora,* the use of 1.0 mg/l IBA also proved to be the best for rooting of regenerated microshoots (Bantawa *et al*., 2009b). As with other medicinal plants, acclimatization of plantlets is an important step for micropropagation of *Picrorhiza* species. Transferred plantlets are maintained in a plastic house, under high humidity (80 % or above) for 8 weeks, and then transferred to larger plastic pots (8 cm 9 10 cm) containing the same potting mixture.

6. DIRECT ADVENTITIOUS (*DE NOVO*) SHOOT ORGANOGENESIS

Recently, Bantawa *et al*., (2011b) developed a reproducible *in vitro* regeneration system of *P. scrophulariiflora* for the first time in this species from leaf derived callus. Induction of more than seven shoot buds per explant was achieved on a Woody Plant Medium (WPM) (Lloyd and McCown 1981) supplemented with 0.1 mg/l NAA and 0.05 mg/l Kin. Shoots elongated when transferred to WPM supplemented with 0.1 mg/l BA, and after transfer to WPM supplemented with 0.1 mg/l NAA, they developed within 2 weeks. Genetic uniformity of these plantlets was confirmed following analysis of leaf tissues using random amplified polymorphic DNA (RAPD) markers analysis.

7. SOMATIC EMBRYOGENESIS

Recently, in *P. scrophulariiflora,* Bantawa (2010) used different explants such as *in vitro* derived roots, leaves, and nodal segments incubated on WPM basal medium supplemented with auxins (0.1–2.0 mg/l of each of 2,4-D and NAA) either alone or in combination with cytokinins (0.05–0.1 mg/l of each of Kin and BA) for embryogenic callus induction. Somatic embryo production differed significantly with respect to the type of explant. Leaf explants developed friable callus along the base of leaf blades. Upon transfer of this friable callus to WPM medium with either Kin or BA (0.1–2.0 mg/l), embryogenic callus was observed, and subsequent transfer of somatic embryos to WPM medium supplemented with ABA (0.1–1.0 mg/l) for 2 weeks, these embryos achieved maturation (92%). Around, 73% of mature somatic embryos germinated in medium containing WPM with 0.5 mg/l Kin and 0.5 mg/l GA3. Synchronization of

maturation of somatic embryos is a necessary prelude for developing whole plants. For *P. scrophulariiflora*, Bantawa (2010) achieved synchronous maturation of somatic embryos from leaf-derived callus by transferring these somatic embryos onto a solidified MS medium containing 0.5 mg/l ABA for 2 weeks, followed by transfer to a fresh MS medium containing 0.5 mg/l Kin for another 4 weeks. Frequency of maturation of somatic embryo differed considerably based on the plant growth regulator (PGR) composition, incubation time on ABA containing medium, and explant source. Conversion of somatic embryos into plantlets often constitutes a limiting step in somatic embryogenesis due to the inability of somatic embryos in certain plant systems to mature (Quoirin 2003). In *P. scrophulariifolra*, somatic embryos did not germinate in PGR free medium. Matured somatic embryos were formed in clusters, each comprising more than ten embryos. These embryos of cotyledonary stage, when separated individually and transferred to medium containing Kin (0.5 mg/l) along with GA3 (0.5 mg/l) germinated at a high frequency (72.84 %) within 4 weeks (Bantawa 2010). Well-rooted plantlets were transferred from culture tubes into plastic cups containing virgin soil mixed with sand (9:1). Over 90 % survival rate was recorded after 8 weeks. Well-rooted plantlets, following acclimatization, were successfully established in the field.

8. SYNTHETIC SEEDS

Somatic embryos of *P. scrophulariiflora* were encapsulated in calcium alginate gel matrix with full liquid strength MS basal medium augmented with a combination of 0.5 mg/l Kin and 0.5 mg/l GA3 (Kinoshita and Saito 1992). A range of sodium alginate levels (2–6 %) was used, and 100 % survival was achieved when beads were stored up to 15 days at 4°C. Interestingly, when the alginate beads were prepared in MS medium containing 0.5 mg/l Kin alone and subsequently stored for 15–45 days, they produced multiple shoots within 10–16 days. Longer storage at lower temperatures resulted in the production of a single shoot per bead. The time required to germinate also increased from 10 to 12 days (at 0-day storage time) to 25 days (at 105-day storage period). Sprouting frequency did not vary between 0 and 105 days storage time, where 100% germination was recorded (Bantawa 2010).

9. STUDIES OF GENETIC DIVERSITY

In general, medicinal plants are valued for their secondary metabolites and hence most attention has been paid to identify the elite lines containing higher amount of secondary metabolites. Plant improvement through breeding depends on the magnitude of the genetic diversity and extent to which this diversity is utilized. Despite the economic and agricultural value of this endangered plant, no attempts have been made to evaluate the potential value of genetic markers in aiding breeding and conservation. Random amplified polymorphic DNA (RAPD) technique is a method of choice for studying genetic diversity for crop species with little or no sequence information available (Nybom 2004). In our previous studies, we found a wide variation of picroside content ranging from 6.35 % to 7.33% (dry weight basis) among the populations of different altitudes (Bantawa *et al.,* 2010). Therefore, present research was aimed to investigate the extent of genetic diversity among these natural populations for devising conservation strategy in *P. scrophulariiflora.*

Bantawa *et al.,* (2012) studied the genetic diversity of *Picrorhiza scrophulariiflora* using RAPD markers where, out of 40 primers screened, five were found to be highly polymorphic, which generated a total 46 bands. Amplicon range from 564 to 2027 bp in size. Simple measure of intra population variability based on the number of polymorphic products scored in a single population over the total number of polymorphic products ranged from a minimum of 30.43 % of the Nepal population to maximum of 76.08 % of East Sikkim and Bhutan population. Being mainly a vegetatively propagated plant, the variation at DNA level may be attributed either to mutations or somatic recombination occurring over time which have been selected and fixed in the population, nevertheless, genetic recombination with a very lower frequency may be contributed.

Since, the number of individual sampled for analysis was small due to extreme rareness of the species, the variation measured perhaps not fully represented the total available genetic diversity of *Picrorhiza*, nevertheless, the percentage of polymorphic bands (86.96%) obtained by RAPD primers in the species was observed very high (Fu *et al.,* 2003). The low percentage variation detected among the Nepal (30.43%) population might be attributed to the very low number of sample analyzed. Thus, germplasm collection mission in future should give special attention for such regions to broaden the genetic basis of gene bank collection and to increase the chance of conserving important genotypes that can be used for improvement programs.

10. FUNCTIONAL GENOMICS

The new emerging area of functional genomics provides huge opportunities to discover novel genes and assign functions to those genes. So far, there are very limited genomic resources available for *Picrorhiza* (Bantawa *et al*., 2012). A total of only 27 ESTs of *P. scrophulariflora* have been deposited at NCBI (www.ncbi.nlm.nih.gov). Recently, Kawoosa *et al*., (2010) have identified two regulatory genes of terpenoid metabolism viz., 3-hydroxy-3-methylglutaryl coenzyme A reductase (pkhmgr) and 1-deoxy-D-xylulose-5-25 phosphate synthase (pkdxs) from another species of *Picrorhiza* i.e., *P. kurroa*. Full length of these genes along with their up-stream sequences have been cloned by rapid amplification of cDNA ends (RACE). This showed the presence of core sequences for light and temperature responsiveness. Electrophoretic mobility shift assay confirmed binding of protein to these motifs. Results have demonstrated that illumination and temperature would play a key role in regulating the expression of pkhmgr and pkdxs, and associated picrosides level in *Picrorhiza*.

Expression of pkhmgr and pkdxs is up-regulated at a low temperature (15°C) and under illumination as compared to a relatively high temperature (25°C) and dark conditions. Picroside contents have exhibited similar trends to levels of gene expression. To rule out the possible limitation of the carbon pool under dark growth conditions, plantlets of *Picrorhiza* were grown in vitro on MS medium supplemented with 3 % sucrose. Similar findings have been observed, wherein up-regulation of both genes accompanying higher picroside contents in the presence of light have been detected in these axenic plantlets.

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