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# Expression Analysis of Key Genes for Picroside-I Production in *in vitro* Grown Shoots of Different Genotypes and Species of Endangered Herb *Picrorhiza*

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***Abstract:*** **Picrorhiza kurroa Royle ex Benth and Picrorhiza scrophulariiflora Pennel are the two highly valued medicinal herbs having hepatoprotective activity along with other medicinal properties. Their medicinal importance is attributed to the presence of two major secondary metabolites, picroside-I (P-I) and picroside-II (P-II). Increasing demand by herbal drug industries and endangered status of this plant necessitates the screening of natural populations for high picrosides content genotypes as well as understanding the molecular biology of picrosides biosynthesis. This is the first comparative study on in vitro propagation and P-I biosynthesis in P. scrophulariiflora and P. kurroa. Micropropagation of different P. kurroa genotypes with varying P-I content was also carried out. Explants were cultured on MS media (Murashige and Skoog, 1962) supplemented with IBA (3mg/l) + KN (1mg/l) + sucrose 3% (w/v) + agar-agar (0.8%) at two different incubation temperatures i.e. 15± 1°C and 25± 1°C and quantified by HPLC for P-I contents. Better growth and higher P-I content was observed at 15± 1°C in comparison to 25± 1°C. This is attempted for the first time where comparative expression profiling of the key genes of MEP and MVA pathway in two species was done. In addition, expression analysis of different P. kurroa genotypes was carried out. Six genes ISPD, ISPE, DXPS, MECPS, HMGS and PMK showed elevated expression levels (~5-20 folds) at 15° C in comparison to 25° C. The genes with elevated expression levels would be useful in understanding the molecular basis of P-I biosynthesis and accumulation for perusal of a systematic genetic improvement programme in Picrorhiza.**

## 1. INTRODUCTION

*Picrorhiza kurroa* and *Picrorhiza scrophulariiflora* are the two important medicinal plant species found in the Himalayan region at an altitude of 2700-4500 meters above sea level. They have been widely used in the traditional as well as modern system of medicine. They are valued as hepato-protective, anti-periodic, cholagouge, stomachic, anti-amoebic, anti-oxidant, anthelmintic, anti-inflammatory, cardio-tonic, laxative, carminative, expectorant, etc. [1-5], [8] and [10-11]. Their medicinal importance is due to the presence of two major secondary metabolites, picroside-I (P-I) and picroside-II (P-II). Due to reckless collection from the wild, lack of organized cultivation and poor seed germination, these plant species have become highly endangered. Increasing demand by herbal drug industries necessitates the screening of natural populations to identify and select superior genotypes having high amount of secondary metabolites. It is essential for the conservation of *Picrorhiza* to encourage ex-situ plantation which require large scale planting material. In view of the problems of conventional propagation and high demand of planting material the large scale multiplication of this species can only be met efficiently and economically in a short span of time by *in vitro* propagation. Generally, secondary metabolites are produced in small amounts in plants naturally. Therefore, it is required to understand the molecular basis of biosynthesis of secondary metabolites so that genetic intervention measures could be planned to increase their contents.

The present work reports comparative study on *in vitro* propagation and P-I biosynthesis in *P. kurroa* and *P. scrophulariiflora* at two different temperatures i.e. 15± 1°C and 25± 1°C. Micropropagation of different *P. kurroa* genotypes with varying P-I content was also carried out. A comparative expression profiling of the key genes of MEP and MVA pathway in two species with respect to P-I content was also done. The key genes were selected on the basis of our previous study [7].

## 2. MATERIALS AND METHOD

### 2.1 Plant Material

Plants of *P. kurroa* and *P. scrophulariiflora* were procured from Himalayan Forest Research Institute, Shimla (H.P.) and NBPGR, New Delhi, respectively and maintained at the polyhouse of Department of Biotechnology and Bioinformatics, Jaypee University of Information Technology, Waknaghat, H.P., India.

### 2.2 Micropropagation of *P. kurroa* and *P. scrophulariiflora*

To prepare explants, shoot apex and nodal regions of *P. kurroa* (PK) and *P. scrophulariiflora* (PS) were excised with a scalpel and washed with distilled water. Different accessions of *P. kurroa* having high (PKS-1), intermediate (PKS-11) and low (PKS-10) P-I content were also taken as identified by our previous study on field grown plants (Shitiz et al. unpublished). Explants were then surface sterilized using 0.5% bavistin and 0.1% mercuric chloride and finally washed with sterile distilled water for 4-5 times. The explants were cultured on MS media [6] supplemented with different concentrations of IBA and KN (ranging from 1-3 mg/l), sucrose 3% (w/v), agar-agar (0.8%) at pH 5.7 and incubated at 25±1°C and 15±1°C under 16 hours photoperiod and 3000 flux light intensity. Rootable shoots were excised from lavishly multiplying shoot cultures having at least 2 cm length and were transferred singly to separate culture jars containing MS Media enriched with rooting growth hormones NAA and IBA of different concentrations. Five replicates of each sample were taken and analyzed as mean ± standard error for number of shoots, shoot length, number of roots and root length after one month of sub culturing.

### 2.3 Quantification of picroside-I (P-I)

Quantification of P-I was done by following the protocol reported by [9] on Waters HPLC system with C18 (5µm) 4.6 x 250mm Waters Symmetry Column and PDA detector (Waters 2996). Fresh shoots were ground in liquid nitrogen and suspended in 80% methanol. The sample mixture was vortexed and kept overnight at room temperature. Following day, the samples were centrifuged at 10,000 rpm for 10-15 minutes and the supernatant was filtered through 0.22µm filter. The filtrate was diluted 10x and injected into above mentioned column. Two solvent systems were used for running the test samples i.e. Solvent A (0.05% trifluoroacetic acid in water) and Solvent B (1:1methanol/ acetonitrile mixture). Solvent A and B were used in the ratio of 70:30 (v/v). The column was eluted in the isocratic mode with a flow rate of 1.0 ml/min. Picroside-1 were detected at absorbance of 270 nm wavelength in a cycle time of 30 min at 30°C. The compounds were identified on the basis of their retention time and comparison of UV spectra with the authentic standards procured from ChromaDex Inc.

### 2.4 Expression analysis using quantitative real time (qRT) PCR

RNA was isolated using Genei™ Raflex total RNA isolation kit as per the manufacturer's instruction. cDNA was synthesized from RNA using Verso cDNA synthesis kit (Thermo Scientific). cDNA was quantified using nanodrop spectrophotometer and concentrations of all samples was adjusted to 100 ng/µl. qPCR was performed using gene specific primers in triplicate on a CFX96 system (Bio-Rad Laboratories; Hercules CA) with the iScript one step RT PCR kit (Bio-rad). The PCR protocol was as follows: denaturation for 5 min at 94°C, followed by 40 cycles each of denaturation for 20 s at 94 °C, annealing for 30 s at 50–65°C, followed by one elongation step for 20 s at 72°C. 26s was used as internal control for normalization.

## 3. RESULTS AND DISCUSSION

### 3.1. *In vitro* grown plants incubated at two different temperatures

Out of tried different media combinations, MS medium supplemented with IBA (3mg/l) + KN (1mg/l) was found to be best combination for multiple shoots and roots formation in *P. scrophulariiflora* and different strains of *P. kurroa* (**Fig. 1**). Plants grown at 15°C were found to be better as compared to 25°C after the period of one month and data was recorded for number of shoots, shoot length, number of roots and root length after one month of sub culturing (**Table 1**).

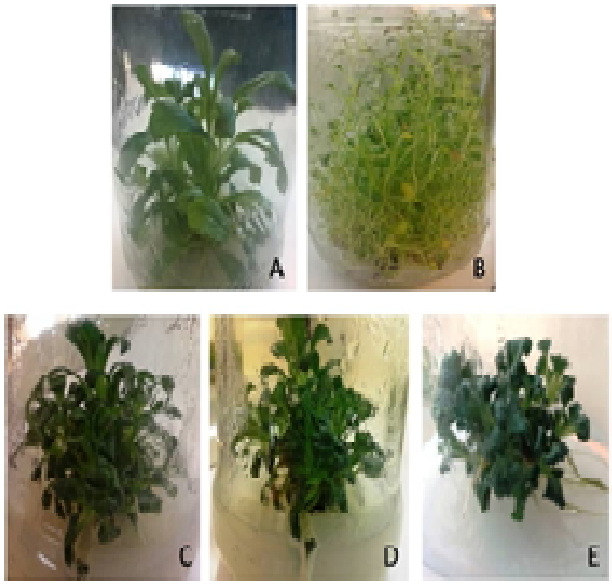


**Figure 1. Cultures growing at 15±1°C on MS media supplemented with IBA (3 mg/L) + KN (1 mg/L). A. *P. kurroa*, B. *P. scrophulariiflora*, C. PKS-1, D. PKS-11, PKS-10**

**Table1. Growth parameters of different strains and accessions at 15±1°C and 25±1°C on MS media supplemented with IBA (3 mg/L) + KN (1 mg/L)**

| | | Growth parameters at 15±1°C | | | | Growth parameters at 25±1°C | | | |
|---|---|---|---|---|---|---|---|---|---|
| S. No. | Species/genotypes | Shoot length (Mean± S.E.) | Shoot number (Mean± S.E.) | Root length (Mean± S.E.) | Root number (Mean± S.E.) | Shoot length (Mean± S.E.) | Shoot number (Mean± S.E.) | Root length (Mean± S.E.) | Root number (Mean± S.E.) |
| 1 | PK | 4.8±0.15 | 25.5±1.15 | 8.7±0.56 | 15.3±1.5 | 4.5±0.78 | 23.3±1.3 | 7.5±0.89 | 12.3±1.6 |
| 2 | PS | 4.3±0.21 | 20.1±1.3 | 8.5±0.23 | 12.1±1.5 | 4.1±0.65 | 18.7±1.5 | 5.3±0.75 | 11.1±1.5 |
| 3 | PKS-1 | 3.5±0.25 | 25.1±0.97 | 8.3±0.54 | 11.5±1.75 | 3.2±0.54 | 16.6±1.5 | 6.5±0.54 | 9.2±0.89 |
| 4 | PKS-11 | 3.15±0.78 | 18.4±1.2 | 7.8±0.81 | 9.2±1.5 | 2.94±0.54 | 14.7±1.1 | 6.6±0.54 | 7.3±1.1 |
| 5 | PKS-10 | 2.92±0.87 | 13.3±1.3 | 7.3±0.54 | 7.1±1.75 | 2.82±0.78 | 11.2±1.3 | 4.9±0.75 | 5.5±1.1 |

### 3.2. Quantification of P-I content at 25°C and 15°C

After the plants were well established and growing well on the shoot multiplication media under in vitro conditions they were analyzed for P-I content. Three different accessions of *P. kurroa* namely PKS-1, PKS-11 and PKS-10 having varying P-I content were also propagated in vitro and quantified for P-I content. It was observed that *P. kurroa* grown under in vitro condition at both 15°C and 25°C showed higher P-I content in comparison to *P. scrophulariiflora*. Similar pattern of P-I content was observed for different accessions at both the temperature conditions. **Table 2** shows the HPLC quantification results.

**Table 2. Ampunt of P-I accumulated in *in vitro* grown shoots of *Picrorhiza***

| S. No. | *Picrorhiza* species/genotype | P-I (µg/mg fresh weight) at 15°C | P-I (µg/mg fresh weight) at 25°C |
|---|---|---|---|
| 1 | PK | 2.64 | 0.29 |
| 2 | PS | 1.42 | 0.18 |
| 3 | PKS-1 | 2.57 | 0.14 |
| 4 | PKS-11 | 1.78 | 0.08 |
| 5 | PKS-10 | 1.42 | 0.04 |

As *P. scrophulariiflora* is having its native place northeastern Himalayas whereas *P. kurroa* having northwestern Himalayas, definitely in natural condition in respective places synthesized metabolites at their best capacity. But when they were cultured in uniform condition slightly higher P-I content in *P. kurroa* is the contribution of different genetic makeup which was confirmed by expression analysis. PKS-1 showed the highest P-I content in field condition in natural environment. It retained its genetic superiority *in vitro* conditions also. Other accessions also showed similar P-I content pattern *in vitro* as their field grown explants.

### 3.3 Expression analysis in relation to P-I content

Expression profiling of key genes of MEP and MVA pathway was done for different tissues of *Picrorhiza* showing variation in P-I content. Eight key genes namely MECPS, DXPS, ISPD, GDS, ISPE, HMGS, HMGR and PMK showing higher transcript abundance in field grown shoots (P-I content 27 µg/mg) in comparison to tissue cultured shoots (having negligible P-I content) were identified from our previous study [7]. The fold expression for all the tissues PK-15, PS-15, PK-25 was calculated with respect to *P. scrophulariiflora* tissue grown at 25°C (PS-25) as it had the least P-I content. Three genes of MEP pathway, MECPS, ISPD and ISPE and one gene of MVA pathway PMK showed ~9-20 folds and ~7 folds higher expression in corroboration with P-I content (**Table 3**).

**Table 3. Fold expression of the genes in PK-15, PS-15 and PK-25 with respect to PS-25**

| Tissue | MECPS | DXPS | HMGR | PMK | ISPD | HMGS | ISPE | GDPS |
|---|---|---|---|---|---|---|---|---|
| ***P. kurroa* 15°C (PK-15)** | 20.73 | 3.41 | 3.11 | 7.24 | 11.6 | 2.89 | 9.28 | 1.6 |
| ***P. scrophulariiflora* 15°C (PS-15)** | 14.35 | 2.61 | 2.88 | 4.23 | 7.39 | 1.34 | 5.11 | 1.51 |
| ***P. kurroa* 25°C (PK-25)** | 3.88 | 1.07 | 1.11 | 2.03 | 3.12 | 1.67 | 2.15 | 1.32 |
| ***P. scrophulariiflora* 25°C (PS-25)** | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |

Expression analysis of the same eight genes was also done for different accessions of *P. kurroa*. Fold expression was calculated for PKS-1 and PKS-11 with respect to PKS-10 having least P-I content. Four genes of MEP pathway MECPS, DXPS, ISPD and ISPE and two genes of MVA pathway HMGS and PMK showed ~9-20 folds and ~5-11 folds higher expression with respect to P-I content at 15°C (**Table 4**). The expression pattern did not showed significant differences at 25°C except for MECPS which showed 6.2 folds higher expression for PKS-1 (**Table 5**).

**Table 4. Fold expression of the genes in PKS-1 and PKS-11 with respect to PKS-10 at 15°C**

| Tissue | MECPS | DXPS | HMGR | PMK | ISPD | HMGS | ISPE | GDPS |
|---|---|---|---|---|---|---|---|---|
| PKS-1 | 19.74 | 9.36 | 3.75 | 11.62 | 15.22 | 5.36 | 10.88 | 4.05 |
| PKS-11 | 14.62 | 5.58 | 3.07 | 8.79 | 13.17 | 4.73 | 4.05 | 3.75 |
| PKS-10 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |

**Table 5. Fold expression of the genes in PKS-1 and PKS-11 with respect to PKS-10 at 25°C**

| Tissue | MECPS | DXPS | HMGR | PMK | ISPD | HMGS | ISPE | GDPS |
|---|---|---|---|---|---|---|---|---|
| PKS-1 | 6.20 | 2.99 | 1.04 | 3.01 | 4.87 | 1.88 | 2.67 | 1.59 |
| PKS-11 | 1.98 | 1.12 | 1.01 | 1.24 | 1.57 | 1.18 | 1.16 | 1.09 |
| PKS-10 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |

From expression analysis results it was observed that more number of MEP pathway genes showed higher expression in comparison to MVA pathway genes vis-à-vis P-I content. It suggests that MEP pathway is playing major role for the biosynthesis of P-I. The results are in corroboration with our previous study [7] which also reported that MEP pathway plays a major role for picrosides biosynthesis.

### 4. CONCLUSION

Our study concludes that better growth and P-I content was observed at 15°C in comparison to 25°C for *P. kurroa* and *P. scrophulariiflora* as well as different accessions of *P. kurroa*. It was also confirmed that the genotype giving highest P-I content under field condition retained its superiority even when grown under *in-vitro* conditions by giving the highest P-I content as compared to the other genotypes. Multiple genes of picrosides biosynthetic pathway showed elevated expression levels vis-à-vis P-I content. These genes would be

useful in understanding the molecular basis of P-I biosynthesis and accumulation for perusal of a systematic genetic improvement programme in *Picrorhiza* for enhancing picroside content.

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