ISSN (Online) 2456 -1304

International Journal of Science, Engineering and Management (IJSEM)
Vol 2, Issue 8, August 2017

Gentipicroside Elicitation in Shoot Cultures of Gentiana kurroo and Swertia chirayita with Molecular Authentication

[1] Payal Kotvi, [2] Rhythem Sharma [3] Satyam Singh, [4] Hemant Sood
Department of Biotechnology and Bioinformatics, JUIT, Waknaghat, Solan, H.P. 173234

Abstract: **-- Gentiana kurroo and Swertia chirayita are critically endangered medicinal herbs which belong to the family Gentianance . They are widely distributed in areas of Jammu and Kashmir, Himachal Pradesh and Uttrakhand and grow between altitude ranges of 1500-3500 m. These two plants have served to be an important source of secondary metabolites i.e gentiopicroside, swertamarin ,amarogentin and maniferin which have high medicinal properties. In the present study an attempt has been made to elicit these phytochemicals by using varied concentration of elicitors like Methyl jasmonate (50-100 µM), Sodium nitroprusside (25-75 µM), Salicylic acid (25-50 µM) and Seaweed (1-3%) in Murashige and Skoog (MS) medium. These phytochemicals were quantified by HPLC and were also observed for their growth and development. The best results were acquired on Seaweed (2%) which gave 0.34% of gentiopicroside in Gentiana kurroo at an interval of 15 days. The other two best results were observed in Salicylic acid (50 µM) i.e 0.20% and Sodium nitropruside (50 µM) i.e 0.19% in Gentiana kurroo at an interval of 30 days. Elicited plants of Swertia chirayita have shown a lower concentration of gentiopicroside i.e 0.1%. The HPLC analysis of shoot cultures grown in MS supplemented with Sodium nitroprusside, Salicylic acid and seaweed elicited cultures revealed enhanced concentration of swertiamarin, amarogentin and manigiferin in Swertia chirayita. This study is the first to utilize Swertia chirayita as a source of gentiopicroside. Since these two plants have been exploited for their metabolites and very few efforts have been made to cultivate them. This study can serve a mean to acquire an enhanced amount of these metabolites to meet the demands of the pharmaceutical sectors. We have also checked the amplification of four genes from conserved pathways (MVA & non- MEP) encoding HMGR, PMK , ISPD and G10H just to authenticate the contribution of key genes for the medicinal compounds biosynthesis so that those could be used for genetic modification for improving the medicinal compounds production.**

Keywords**: Gentiana kurroo, Swertia chirayita, Elicitation, Gentiopicroside and HPLC.**

I. INTRODUCTION

Medicinally important herbs occupy the biggest sector of the pharamceutical industry. Their ability to synthesis certain chemical compounds which fall under the category of primary and secondary metabolites, where on one aspect serve as their natural mechanism against any sought of environmental stress and on the other aspect play their role by becoming part of various medicinal formulations.

Gentiana kurroo Royle and *Swertia chirayita* are highly endangered bitter herbs of Himalayan origin which belong to the family Gentianaceae. They are natives of North-western Himalayas and enormously found in places like Jammu & Kashmir, Himachal Pradesh, and Uttrakhand at an altitudes of 1500-3400 m. The two plant species are an abundant source of medicinally important secoirridoid glycosides (gentiopicroside, swertamarin, amarogentin), alkaloids etc which serve in treating skin diseases, digestive disorders etc. They have also shown to exhibit antibacterial, antifungal, antiviral, anticancer, anti-inflammatory and antidiabetic activities. Over exploitation and utilization of resources has lead to their extinction. Therefore, the red data book of Indian plants has listed these species as endangered and its status as critical. Major threats for this species are loss of habitat and unregulated harvesting. The population of the species has declined due to illegal collection and industrialization. Due to rapid deforestation change in climate has also added to the deteriorating condition. In both the species seeds offer limitations in their use due to low viability, small size and low germination percentages. There are few reports available on application of systematic agro-techniques for improving their cultivation and reclamation of their natural population. So in the present study we have used in vitro grown shoots of S. chirata and G.kurroo and grow them in optimized MS media supplemented with different elicitors which directly or indirectly contribute in the biosynthetic pathways of their secondary metabolites production which are of medicinal importance (Varun et al 2013). This study

ISSN (Online) 2456 -1304

International Journal of Science, Engineering and Management (IJSEM)
Vol 2, Issue 8, August 2017

also helps in generating scope of developing strategies for genetic improvement to positively effect the biosynthetic machinery. To overcome this challenge 4 genes of the biosynthetic pathway (MVA & non-MEP) encoding HMGR, PMK, ISPD, G10H which were reported by Pradhan et al(2015) and amplified on elicited shoots of both the plants. Therefore the present study generates a lead in identifying the genes which contributes for gentipicroside, swertamarin and amarogentin especially in G.kurroo and S.chirayita.

II. MATERIAL AND METHODS

2.1 Collection of plant material

Swertia chirayita and Gentiana kurroo plants were acquired from Himalayan Forest Research Institute, Himachal Pradesh, Green house grown plants of Swertia chirayita and Gentiana kurroo were raised and maintained in Jaypee University of Information Technology, Waknaghat, Himachal Pradesh, India. The in vitro plantlets of Swertia chirayita and Gentiana kurroo which were maintained in the culture room (25°C ± 2) with light intensity (5,800 W m-2) and relative humidity (≈75%) with photoperiod of (16 hrs. day/8 hrs. night).Tissue samples from the invitro grown plants were taken for elicitation and gene amplification.

2.2 Optimization of culture condition for elicitation in shoot cultures of Gentiana kurroo and Swertia chirata

Invitro grown shoots for the two species were taken and cultured on MS media supplemented with growth hormones and elicitors. Shoot explants of Swertia were cultured on MS media containing IBA(1mg/l)+KN(3mg/l) where on the other hand shoot explants of Gentiana were established on the MS media containing IBA(1mg/l)+KN(3mg/l) with 15 grams of sucrose and 8 grams of agar. Both the cultures were supplemented with elicitators viz. Methyl jasmonate, salicylic acid, SNP, and seaweed.(Table 1) The elicitators were filter sterilised using 0.22 µm sterile filter. The cultures were kept for optimization under (25±2)ºC light conditions for 30 days.

Table 1. Concentration of different elicitors used for invitro shoot cultures of G.kuroo and S.chirayita

S.NO	Elicitors	Concentration
1	MeJa	50, 75,100 uM
2	SNP	25,50,75 uM
3	Salicylic acid	25,50 uM
4	Seaweed	1% 2% 3%

2.3 Data collection

The elicitated and control shoots were collected at an interval of 15 and 30 days of their culturing. The data was collected for number of shoots formation shoot biomass and gentiopicroside production by carrying out HPLC analysis.

2.4 Quantification by HPLC

The plant materials (100 mg) of the two species supplemented with elicitors were ground using motor pestle with liquid nitrogen and were thereafter suspended in 80% methanol for overnight incubation. The samples were then centrifuged at 10,000 rpm for 15 min. Supernatent was then filtered through 0.22 µm filter. The desired separation was obtained by implying a gradient method where mobile phase A composed of 0.1% TFA in water and mobile phase B was mixture of acetonitrile : water (70:30). The linear gradient at a flow rate of 1.0 ml/min was start with 15% B; 20% B in next 5 min, 70% B in next 25 min, hold for 5 min; 15% B in next 5 and equilibrated for 5 min at 240 nm UV wavelength. The phytochemical gentipicroside was separated and recognized based on their retention time (RT) and UV-spectra assessment.

2.5 RNA isolation and preparation of cDNA

RNA was isolated from the elicited tissues of S. chirata and Gentiana kurroo using Trizol Reagent (Ambion Inc., USA). The isolated RNA was quantified using spectrophotometric (A260 and A280) measurements (NanoDrop, Thermo Scientific, USA). Further integrity of the RNA was assessed on agarose gel stained with 1% (w/v) ethidium bromide. 100 µg of RNA was taken to prepare cDNA using verso cDNA kit using verso oligo dT and verso RT enhancer, and visualized the samples on 1% ethidium bromide stained gel.

2.6 Identification of secondary metabolic pathway genes in Swertia chirayita and Gentiana kurroo.

The metabolic route for synthesis of gentiopicroside i.e a seco-irridoid involves the MVA, MEP, secoiridoid

ISSN (Online) 2456 -1304

International Journal of Science, Engineering and Management (IJSEM)
Vol 2, Issue 8, August 2017

and phenylpropanoid route. Six different genes from the MVA and MEP pathway were finalised based on the previous conducted studies. The primers of the selected genes were procured from Padhan et. al. 2015.These primers were used for the gene expression analysis in samples of Gentiana kurroo and Swertia chirayita elicited plants.

III. RESULTS

The shoot explants of Gentiana kurroo and Swertia chirayta cultured on MS media supplemented with plant growth elicitors under (25 ± 1)°C were observed in the interval of 15 days (Fig. 3[a] and 3[b]). The 0 day sample were also collected. After 30 days final sampling was conducted and fresh weight of the samples of both species was recorded (Fig. 3[c] and 3[d]). The data was collected for observing the growth, developmental and morphological status of invitro grown shoots in Swertia chirayita and Gentiana kurroo along with the gentiopicroside quantification.

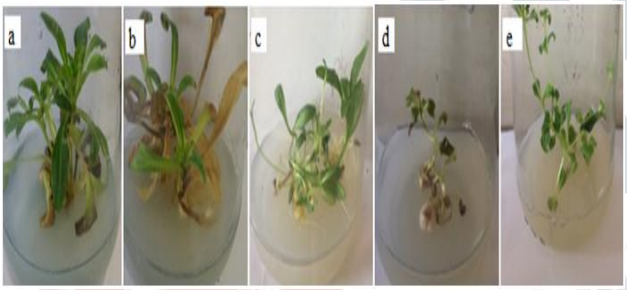

Fig. 3[a]: Effect of elicitors on growth and development in Swertia chirayita after 15 days (a) Control (b) Methyl jasmonate(c) SNP (d) Salicylic acid (e) Seaweed

Fig. 3[b]: .Effect of elicitors on growth and development Gentiana kurroo after 15 days (f) Control (g) Methyl jasmonate (h) SNP (i) Seaweed (j) Salicylic acid

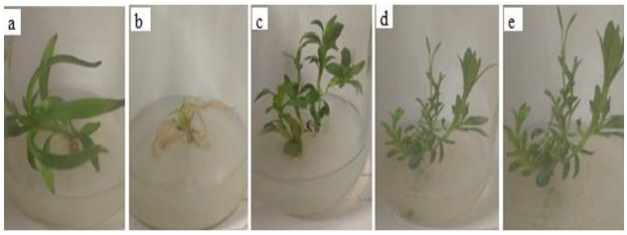

Fig. 3[c]: Effect of elicitors on growth and development Gentiana kurroo after 30 days (a) Control (b) Methyl jasmonate (c) SNP (d) Seaweed (e) Salicylic acid

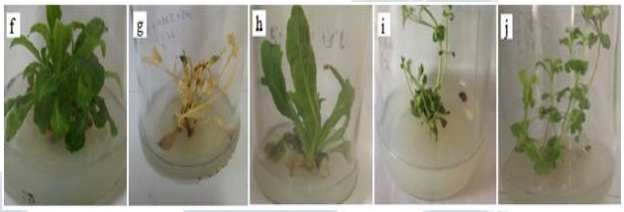

Fig. 3[d]: Effect of elicitors on growth and development in Swertia chirayita after 30 days (f) Control (g) Methyl jasmonate (h) SNP (i) Salicylic acid (j) Seaweed

3.1 Phytochemical analysis

HPLC analysis of the elicited plants of Gentiana kurroo and Swertia chirayita have revealed the effect of the elicitors on the content of the secondary metabolite gentiopicroside. Gentiopicroside content varied from 0.01-0.34% of fresh weight in the two above mentioned species. The highest level of gentiopicroside has been accumulated in the invitro grown shoot cultures of Gentiana kurroo which was elicited by seaweed i.e 0.34% of fresh weight within 15 days of incubation. Whereas no elevation has been noticed when compared with the standards. Plants elicited by SA accumulated 0.20% of fresh weight in 30 days. Elevated levels of gentiopicroside seems to be accumulated in elicited plants Gentiana kurroo with SNP. A considerable decrease was witnessed in Gentiana kurroo elicited with seaweed and seaweed elicited cultures within 30 days of incubation. The decrease in metabolite content might be due to feedback inhibition or rechannaleization 3.1.[A] and Fig 3.1.[B]). On the other hand, invitro grown elicited plants of Swertia chirayita have accumulated a constant range of

ISSN (Online) 2456 -1304

International Journal of Science, Engineering and Management (IJSEM)
Vol 2, Issue 8, August 2017

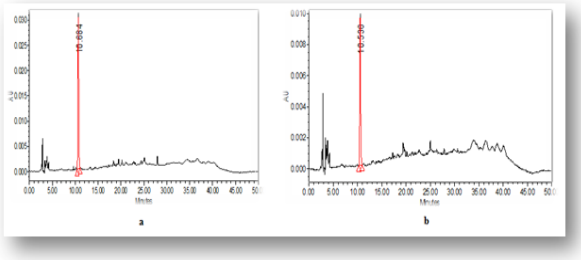

Fig 3.1.[A]: HPLC analysis of seaweed elicited shoots of Gentiana kurroo plant elicitor (a) After 15 days (b) After 30 days

of metabolic flux of their biosynthetic pathways. Whereas SA acid have shown to increase the metabolite content within 15 days of incubation (Fig gentiopicroside i.e 0.1% of fresh weight. No elevation has been found in any of the elicited plants of Swertia chirayita.

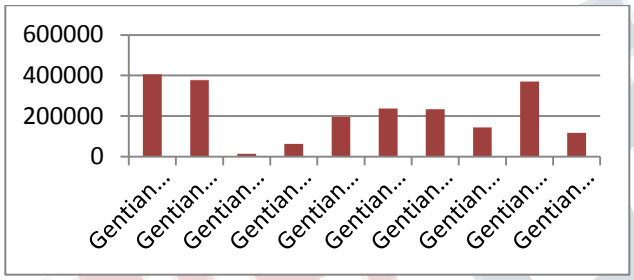

Fig.3.1.[B]: Quantification of gentiopicroside in Gentiana kurroo

3.2 RNA isolation

RNA of the quantified samples were extracted using TRIzol method. Quantity, concentration and purity of the RNA was estimated by spectrophotometric measurements at A260 and A280 nm . The samples were further visualised on the ethidium bromide gel.

3.3 cDNA synthesis

100 µg of RNA was taken to prepare cDNA using verso cDNA kit, verso oligo dT and verso RT enhancer. A band of 525 base pair was visualised after running the samples on 1.2% ethidium bromide stained gel. Quantity, purity and concentration of RNA was estimated by spectrophotometric measurements at A260 and A280 nm.

3.4 Identification of pathway genes for Gentiana kurroo and Swertia chirayita

To understand the biogenesis of the gentiopicroside production, 4 genes were selected based on their role in mangiferin, amarogentin and swertiamarin biosynthesis in Swertia chirayita as described in Padhan et al. 2015. The primers of the selected genes were procured from Pradhan et al. 2015.These primers were used for the gene amplification in samples of elicited plants of Gentiana kurroo and Swertia chirayita.(Fig.3.4)

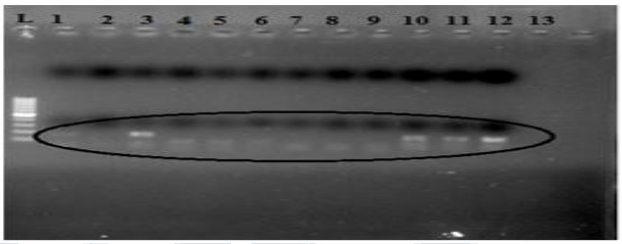

Fig.3.4: Gene amplification in Gentiana kurroo (a) L –Ladder (b) 1-3 HMGR (c) 4-6 MVK (d) 7-9 ISPD (e)10-12 G10H

IV. DISCUSSION AND CONCLUSION

Gentiana kurroo and Swertia chirayita have been explored for their medicinally important aspects contributing in the pharmaceutical sector. These species have been studied at their molecular and genetic levels to understand their biogenesis pathways concerning production of secondary metabolites. Attempts have been made to elicit these secondary metabolites by using elicitors like MeJA, SA, SNP and seaweed. In the past studies different concentrations of MeJA (20-50 µM) have shown to enhance the levels of anthocyanin in the roots of Gynuria bicolour [1]. Similiar studies on the use of MeJA (0.5 µM) as an elicitor has also shown to enhance the anthocyanin content by 2-3 folds and Vacinium phallaea over its control [2]. To the contrary, in our present study MeJA (75 µM) has shown to exhibit tissue browning followed by inhibitory effects on the gentiopicroside production in both plant species genus. SA has also been a potential elicitors to study the variability of the compounds with its different concentrations. In a study conducted earlier SA has not been seen to contribute towards the increase in the anthocyanin content [1]. SA acid (20 µM-500 µM) has shown to induce inhibitory effects on the ATP synthesis in tobacco cultures[3]. SA in concentration of 50 ppm has shown to enhance seed germination in the groundnut cultivars [4]. With respect

to our present study SA (50 µM) acid has not shown any conciderable increase in the gentiopicroside content in Gentiana kurroo with respect to the control. The quantification analysis of the effects of SA on Swertia chirayita to enhance gentiopicroside content has been reported for the first time. Earlier the plant has only been utilised for its potential secondary metabolites as swertiamarin, amarogentin and mangiferin. Salicylic acid has also contributed towards enhancing the content of procyanidin and anthocyanin in cultured grape cells. In the same study positive effects have also been observed towards enhancing the enzyme activity of phenylalanine ammonium lyase and chalconeflavanone isomerase which contribute towards photochemical production [5]. Studies were also conducted with combinations of plant elicitors such as MeJA and SA contributing towards taxol production [6]. It was observed that MeJA and SA has strongly increased anthraquinone accumulation in transgenics and non-transgenics [7]. In a study conducted SA (50 µM) has enhanced the production of hypericin (7.98-fold) and pseudohypericin (13.58-fold) in Hypericum hirsutum [8]. Effect of seaweed has also been seen to enhance the metabolite content. Seaweed (2%) has shown 3.23, 1.55, 2.42, 2.52 and 2.41 folds enhancement in the various tissues of the plant species Picrorihza kurroo. Studies have also indicated to stimulate P-I production by 2.60, 2.01, and 1.35-fold by using SNP and seaweed [9]. Similarly in our present study seaweed has contributed towards the maximum gentiopicroside concentration (0.31%) in Gentiana kurroo but no additive enhancement with respect to control. Further more to the study their has been a constant effect of the gentiopicroside content (0.1%) in Swertia chirayita.

Another aspect of the present study was molecular characterisation of the two plant genus for gentiopicroside production in Swertia chirayita and Gentiana kurroo was carried out where padhan et.al has identified 16 genes in sweria chirata for the production of marker compounds. Genes (HMGR, MVK, ISPD , G10H) from the conserved pathways were selected in the present study for gentiopicroside production and characterization[10]. Further amplification of these genes were carried out on Gentiana kurroo and Swertia chirayita elicited cultures to understand its biogenesis of gentiopicroside production. Amplification of HMGR gene was observed in seaweed elicited shoot culture at 0.31% concentration in Gentiana kurroo. Whereas there was no HMGR amplification visualized for Swertia chirayita. Respectively the other 4 gene were amplified on Gentiana kurroo and Swertia chirayita elicited shoot cultures. Amplification of G10H was seen with respect to SNP, seaweed, and SA elicited shoot cultures in Gentiana kurroo.

So, this study infers that the selected conserved pathway genes contributing for gentiopicroside production in Gentiana kurroo showed no amplification for HMGR, MVK ,ISPD and G10H in elicited shoots of Swertia chirayita .It must have other genes which needed to be explored for further experimentation.

REFERENCES

1. Y. Shimizu, K. Maeda, M. Kato and K. Shimomura, "Co-expression of GbMYB1 and GbMYC1 induces anthocyanin accumulation in roots of cultured Gynura bicolor DC. plantlet on methyl jasmonate treatment", Plant Physiology and Biochemistry, vol. 49, no. 2, pp. 159-167, 2011.

2. Y. Fang, M. Smith and M. Pepsin, “Effect of exogenous Methyl jasmonate in elicited anthocyanin producing cell cultures of Ohelo (Vaccinump ahalae)” , In Vitro Cell.Dev.Biol, pp. 106-113, 1998.

3. M. Rivas-San Vicente and J. Plasencia, "Salicylic acid beyond defence: its role in plant growth and development", Journal of Experimental Botany, vol. 62, no. 10, pp. 3321-3338, 2011.

4. War and H. Sharma, "Effect of jasmonic acid and salicylic acid induced resistance in groundnut onHelicoverpa armigera", Physiological Entomology, vol. 39, no. 2, pp. 136-142, 2014.

5. N. Obinata, T. Yamakawa, M. Takamiya, N. Tanaka, K. Ishimaru and T. Kodama, "Effects of Salicylic Acid on the Production of Procyanidin and Anthocyanin in Cultured Grape Cells", Plant Biotechnology, vol. 20, no. 2, pp. 105-111, 2003.

6. N. Chaichana and S. deeranupattana, “Effects of Methyl Jasmonate and Salicylic Acid on Alkaloid Production from in vitro Culture of Stemona” , International Journal of Bioscience, Biochemistry and Bioinformatics, Vol. 2, No. 3, pp. 146-150, 2012.

7. V. Bulgakov, G. Tchernoded, N. Mischenko, M. Khodakovskaya, V. Glazunov, S. Radchenko, E. Zvereva, S. Fedoreyev and Y. Zhuravlev, "Effect of salicylic acid, methyl jasmonate, ethephon and cantharidin on anthraquinone production by Rubia

cordifolia callus cultures transformed with the rolB and rolC genes", Journal of Biotechnology, vol. 97, no. 3, pp. 213-221, 2002

8. Coste, L. Vlase, A. Halmagyi, C. Deliu and G. Coldea, "Effects of plant growth regulators and elicitors on production of secondary metabolites in shoot cultures of Hypericum hirsutum and Hypericum maculatum", Plant Cell, Tissue and Organ Culture (PCTOC), vol. 106, no. 2, pp. 279-288, 2011.

9. N. Sharma and V. kumar, "Modulation of Picroside - I Biosynthesis in Grown Elicited Shoots of Picrorhiza kurroa In Vitro", Journal of Plant Growth Regulators, 2016.

10. D. Klessig and J. Malamy, "The salicylic acid signal in plants", Plant Molecular Biology, vol. 26, no. 5, pp. 1439-1458, 1994.

11. K. Yazaki, K. Takeda and M. Tabata, "Effects of Methyl Jasmonate on Shikonin and Dihydroechinofuran Production in Lithospermum Cell Cultures", Plant and Cell Physiology, vol. 38, no. 7, pp. 776-782, 1997.

12. M. Traw, "Interactive Effects of Jasmonic Acid, Salicylic Acid, and Gibberellin on Induction of Trichomes in Arabidopsis", PLANT PHYSIOLOGY, vol. 133, no. 3, pp. 1367-1375, 2003.

13. Khan, M. Waqas, M. Hamayun, A. Al-Harrasi, A. Al-Rawahi and I. Lee, "Co-synergism of endophyte Penicillium resedanum LK6 with salicylic acid helped Capsicum annuum in biomass recovery and osmotic stress mitigation", BMC Microbiology, vol. 13, no. 1, pp. 51, 2013.

14. G. Sudha and G. Ravishankar, "Elicitation of anthocyanin production in callus cultures of Daucus carota and the involvement of methyl jasmonate and salicylic acid", Acta Physiologiae Plantarum, vol. 25, no. 3, pp. 249-256, 2003.

15. R. Chaudhuri, A. Pal and T. Jha, "Production of genetically uniform plants from nodal explants of Swertia chirayita Buch.-Ham. ex Wall.—an endangered medicinal herb", In Vitro Cellular & Developmental Biology - Plant, vol. 43, no. 5, pp. 467-472, 2007.

16. J. Ueda and J. Kato, "Inhibition of cytokinin-induced plant growth by jasmonic acid and its methyl ester", Physiol Plant, vol. 54, no. 3, pp. 249-252, 1982

17. J. K. Padhan, V. Kumar, H. Sood, T. R. Singh, R. S. Chauhan. "Contents of therapeutic metabolites in Swertia chirayita correlate with the expression profiles of multiple genes in corresponding biosynthesis pathways", Phytochemistry, vol. 116, pp. 38-47, 2015.