EGYPTIAN JOURNAL OF BOTANY (EJBO)

Print ISSN: 0375-9237
Online ISSN: 2357-0350

SPECIAL ISSUE:
Environmental Botany and Microbiology

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PUBLISHED BY

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SHORT COMMUNICATION

Gentiopicroside (GPD) is a major secoiridoid glucoside in plants of the family Gentianaceae, predominantly in the genus *Gentiana*. The roots and shoots of *Gentiana kurroo* Royle, indigenous to the Indian Himalayas are rich in GPD. It is used extensively for treating various diseases in Ayurvedic medicine. In this study, *in vitro* shoot cultures of *G. kurroo* were stimulated with *Trichoderma viride, Aspergillus niger, Fusarium oxysporum,* and an endophytic fungal cell wall extract to enhance the production of GPD. The results showed that treatment with the endophytic fungal extract produced the highest concentration of GPD (30.4 mg/g DW) than other treatments after 48 hours. The endophytic fungus was identified as *Parengyodontium album*. This study provides a simple and efficient method for *in vitro* elicitation of GPD.

Keywords: fungal elicitors; endophyte; *Gentiana kurroo*; gentiopicroside; *in vitro* shoots

ARTICLE HISTORY
Submitted: November 28, 2023
Accepted: September 05, 2024

CORRESPONDANCE TO
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DOI: 10.21608/ejbo.2024.240513.2582

EDITOR
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INTRODUCTION

The roots and rhizomes of *Gentiana kurroo* contain a large concentration of gentiopicroside (GPD), a secoiridoid glucoside, with a lower concentration found in the leaves possesses important pharmacological activities, including anti-inflammatory, anti-arthritis, antibacterial, anticancer, anti-osteoporotic and hepatoprotective properties. GPD induces apoptosis in human ovarian carcinoma cells (SKOV3) and HeLa cell lines through the mitochondrial apoptotic pathway and also arrests the cell cycle arrest at the G2/M phase (Hu *et al.*, 2021; Li *et al.*, 2019). Recently, an *in vivo* evaluation of GPD in experimental mice showed that has osteogenesis proprieties and influences the bone morphogenetic protein 2 (BMP2) and Wnt/β-catenin pathways (Jiang *et al.*, 2021). It also showed anti-arthritis activity by activating the NLRP3 inflammasome in acute gouty arthritis mice (He *et al.*, 2021). In Ayurvedic medicine, *G. kurroo* has been used widely for treating various diseases in India.

This herb grows over 1600–3500 Masl and is endemic to the Himalayan region and extensively harvested from wild habitat for its immense medicinal properties. The unregulated collection depleted its natural population drastically over the years. It is listed as a critically endangered species by the International Union for Conservation of Nature (IUCN). Due to its poor seed germination and slow growth, the plant is not cultivated. Several *in vitro* techniques have been established to propagate the plant under controlled conditions (Fiuk and Rybczyński, 2007; Badrelden, 2017; El-Shafey *et al.*, 2019). A few studies have assessed the production of GPD under *in vitro* conditions (Alphonse and Thiagarajan, 2021; Alphonse *et al.*, 2021, 2022). Therefore, it is worthy to find novel approaches to improve the yield of GPD in *G. kurroo*.

Elicitor molecules derived from biological origin especially the extracts of fungi and seaweeds are frequently used in plant tissue culture to elicit valuable secondary metabolites (Halder *et al.*, 2019; Sharma *et al.*, 2015; Darwish and Ahmed, 2020). Fungal cultures have been identified as the most effective elicitors for secondary metabolite production in *in vitro* plant cultures (Salehi *et al.*, 2019, 2020; Farhadi *et al.*, 2020; Sák *et al.*, 2021).

Fungal endophytes coexist symbiotically in plant tissues and are effective in stimulating the production of secondary metabolites by the host. Endophytes, in contrast to harmful fungi, promote a mutually beneficial connection without harming the host (Wen *et al.*, 2022), which are found in nearly all plants, including those that have invaded the Arctic, Antarctic, deserts, seas and tropical rainforests (Jin *et al.*, 2021; Liu-Xu *et al.*, 2022). *Fusarium oxysporum* (Alallaf *et al.*, 2023), *Aspergillus niger* (Aziz *et al.*, 2021; Lubna *et al.*, 2018), *and Trichoderma viride,*

have been reported as endophytes in many plants and regulate plant metabolism.

Plants have developed sensors to detect distinct microbial chemicals, generally called microbe-associated molecular patterns (MAMPs). Chitin, a key structural building component of the fungal cell wall, is the most well-studied fungal MAMP (Hématy *et al.*, 2009; Fesel and Zuccaro, 2016). It was also reported that the fungal cell wall fragments serve as signals to activate the genes involved in synthesizing plant defence chemicals (Tashackori *et al.*, 2018). Therefore, few selected fungal cultures were used to elicit the GPD in *G. kurroo* plant material.

In this study, we report the efficacy of some fungal species and endophytic fungi isolated from *G. kurroo* to elicit GPD. The objectives of this study are 1) to isolate and characterize the endophytic fungus from *G. kurroo,* and 2) to compare the elicitation efficacy of endophytes and other known species.

MATERIALS AND METHODS

Plant materials

Plant materials were collected from Solan, Himachal Pradesh, and authenticated by Prof. Raina., Dr. Y. S Parmar, University of Horticulture and Forestry, Solan, Himachal Pradesh, India.

In vitro shoot culture maintenance

The *in vitro* shoots were establishment as described in our previous work Alphonse *et al.* (2021). Briefly, the apical meristem was detached from mother plant, then aseptically cleaned with 1% sodium hypochlorite and 0.1% mercury chloride. After rinsing four or five times in sterile distilled water, the surface-sterilized explants were placed on basal MS medium (Murashige and Skoog 1962) supplemented with 1 mg/L 2, 4-D, and 0.5 mg/L Kn for callus establishment. These calli were then cultured on the liquid MS medium fortified with 2 mg/L IBA and 1 mg/L IAA with shaking on a Gyratory shaker at 100 rpm at 24°C for somatic embryo formation. Regenerated shoots were multiplied on MS medium fortified with 2 mg/L BA and 0.5 mg/L IAA. All the cultures were maintained under 16 h photoperiod at 40 mmol m^{-2} s^{-1} at 24°C. All the plant growth regulators (PGRs) were purchased from Sigma-Aldrich (Mumbai, India) while basal salts were purchased from Himedia (Mumbai, India).

Endophyte isolation

Young leaves of *G. kurroo* were collected in humidified zip-lock bags and transported to the laboratory from Solan, Himachal Pradesh. The endophytic fungus was isolated in the same manner described in previous reports with a few changes (Govindappa *et al.*, 2011; Premalatha and Kalra, 2013). The leaves were washed in running water for about 30 minutes to remove the dust and dirt particles and then dipped in 1% Bavistin solution for 10 minutes. After washing in running water, the leaves were surface sterilized with 70% alcohol for 30 sec followed by 5 min treatment with 1.0% sodium hypochlorite (NaOCl), then washed twice in sterile distilled water. The sterile leaves were cut into 0.5 cm x 0.5 cm long pieces with a sterile blade and cultured in potato dextrose agar (PDA) medium containing 50 mg/mL streptomycin sulphate and chloramphenicol. The culture conditions were maintained at 25±2°C until visible fungal hyphae development. The cultures were subcultured onto fresh PDA media at 10-day intervals to carry out morphological and molecular characterization.

Characterization of the endophyte
Molecular analysis

The endophytic fungus isolated from the leaves of *G. kurroo* was identified using molecular techniques described in a previous study (Hemmati *et al.*, 2020). The genomic DNA of the endophytes was amplified in a polymerase chain reaction (PCR) with the universal internal transcribed spacer (ITS) primers (ITS1 and ITS4) (White *et al.*, 1990). The ITS1 (TCC GTA GGT GAA CCT GCG G) and ITS4 (TCC TCC GCT TAT TGA TAT GC) primers were used to amplify the non-coding region of ribosomal RNA (rRNA) genes between 18S, 5.8S, and 28S (Manter and Vivanco, 2007). The amplified PCR products were sequenced and analyzed by Joat Yaazh Xenomics, Coimbatore, India. The sequence data was compared with the full-length ITS fragments published using the Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/blast/) database. The total score and maximum homology were used to assign the neighboring strains having maximum percentage match in the GenBank database.

Fungal extract preparation and elicitation

The fungal cultures of *Trichoderma* sp., *Aspergillus* sp., and *Fusarium* sp. procured from NCCS, Pune, India, and the endophytes isolated from *G. kurroo* were established on PDA broth in 250 mL conical flasks. The culture flasks were maintained at 30 ±2°C for 10 days on an orbital shaker (150 rpm). The culture flasks were autoclaved at 121°C at 15 psi/g for 20 min and the fungal mat was filtered through a cheesecloth and dried at 50°C for 48 hr in an oven. The well-dried mycelia were ground to a fine powder using a mortar and pestle. This powder was suspended in sterile

water at a concentration of 10 g/L. This solution was then used for the elicitation experiments. Approximately 5 to 6 cm long shoots which were previously established on solid media, were inoculated into 250 mL Erlenmeyer flasks containing 15 mL of liquid MS media supplemented with 2 mg/L BA and 0.5 mg/L IAA. The flasks were maintained for 35 days on a Gyratory shaker at 90 rpm under 40 mmol m^{-2} s^{-1} at 23°C, which was then used for elicitation. A preliminary experiment was conducted with 2 mL/flask concentration of fungal homogenate for up to 5 days to find out the toxicity; since the cultures showed a lethal effect, the treatments were restricted to 48 hrs with different concentrations of fungal homogenate (1.0, 1.5, 2.0, 2.5, and 4.0 mL per flask).

Estimation of gentiopicroside

The whole shoots including the leaves and stem were dried at 50 ºC using hot oven for 2 days. The dried *in vitro* samples were pulverized in a mortar and pestle. GPD was isolated and analyzed using the method described by Alphonse and Thiagarajan (2021). HPLC was done in a Shimadzu Corporation instrument (Kyoto, Japan), which was equipped with a CBM-20A system, binary LC-20AP pump, and SPD-M20A Photo Diode Array (PDA) detector. Chromatographic separation was accomplished using a Shimadzu Shim-pack GIST C18 column (250 mm × 4.6 mm ID., 5 µm particle size). A one percent acetic acid in water-methanol at 70:30 (v/v) solvent combination was used as the mobile phase. The solvents were vacuum filtered via a 0.25 µm nylon membrane after degassing with an ultrasonic sonicator bath (Sonica 2400 S3, Milan, Italy) (Merck). A SIL-20AC HT Autosampler (Shimadzu) was used to inject 10 µL of the sample precisely. The GPD separation efficiency was compared using isocratic elution at 1 mL/min flow. The chromatogram peaks were detected by comparing the RT and UV spectra of real GPD. LC-Solution tools were used to evaluate the collected data (Shimadzu Corporation).

Statistical analysis

The elicitor treatments were conducted in triplicate, and the observed data set was analyzed with SPSS software version 16.0. The significant differences among the treatments were undertaken statistically using one-way ANOVA by comparing the means using Duncan's multiple range test. Statistically, the groups are considered significantly different at $P \leq 0.05$.

RESULTS AND DISCUSSION

Endophytic fungus isolation and identification

A white mycelium was observed at the cut portion of inoculated leaves of *G. kurroo* after 8 days on PDA media. The mycelium was used for further subculture and genomic analysis. The endophyte was identified as *Parengyodontium album* by genome sequence analysis of ITS regions of rDNA. The phylogenetic tree developed from the sequence data showed 99% similarity to the GenBank data (Figure 1).

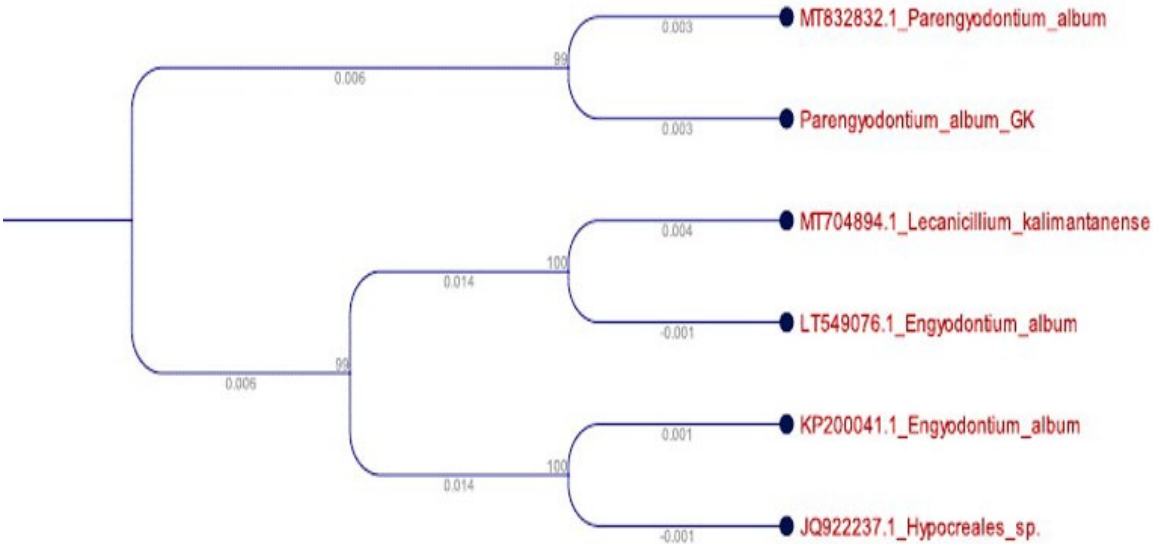

Figure 1. Phylogenetic tree based on neighbor-joining analysis of the ITS sequence of the endophytic fungi obtained from *G. kurroo*

Elicitation of *G. kurroo* shoots and GPD analysis

The *in vitro* shoots of *G. kurroo* were induced with four fungal extracts to enhance the biosynthesis of GPD. The four fungal extracts influenced the amount of GPD produced. GPD levels of 23.2, 19.2, 20.8 and 30.4 mg/g which is 224.3%, 167.59%, 190.22% and 324.74% higher than the control in response to *T. viride, A. niger, F. oxysporum,* and endophytic fungal extracts, respectively, after 48 hr of treatment. In 24-hour treatments, GPD accumulation varied with increasing concentrations of fungal extracts and the highest concentration was observed at 4 mL treatment for all four fungi (Figures 2 and 3).

A previous study showed that elicitation of *Vitis vinifera* cell cultures with cellulase from *T. viride* and cell wall extracts of *F. oxysporum* enhanced stilbenes production by 39 and 2.5 times (Sák *et al.*, 2021). The cell cultures of *Corylus avellana* were elicited with mycelial extract and cell filtrate obtained from the fungus (*Camarosporomyces flavigenus*), either singly or in combination, were used to increase paclitaxel production. About 4.8-fold higher paclitaxel accumulation was found in the combined treatment of cell extract and culture filtrate than in individual applications (Salehi *et al.*, 2020). Similarly, enhanced paclitaxel production was achieved in *Corylus avellana* cell culture exposed to the endophytic fungi (*Epicoccum nigrum* strain YEF2), which produced 5.5 higher yields than the untreated samples (Salehi *et al.*, 2019). Similarly, the *C. avellana* cell cultures treated with *Coniothyrium palmarum* (strain YEF_{33}) isolated from *Taxus baccata* produced as much as 5.8-fold higher paclitaxel when combined with methyl-β-cyclodextrin (Farhadi *et al.*, 2020).

The fungal cell wall promoted the production of the phenylpropanoid pathway-derived compounds in the hairy roots of *Linum album* (Tashackori *et al.*, 2018). A similar reason might have caused the overproduction of GPD in the *G. kurroo* shoots. The diverse response of shoot cultures to various fungal elicitors in increasing GPD biosynthesis may be attributed to unique interactions between the fungi and plant cells (Somssich and Hahlbrock, 1998). Therefore, this fungal elicitation method can be an effective tool to increase the production of GPD in the *in vitro* shoots of *G. kurroo*.

CONCLUSION

This study demonstrated that fungal cell wall homogenates can enhance the GPD content of *G. kurroo in vitro* shoots.

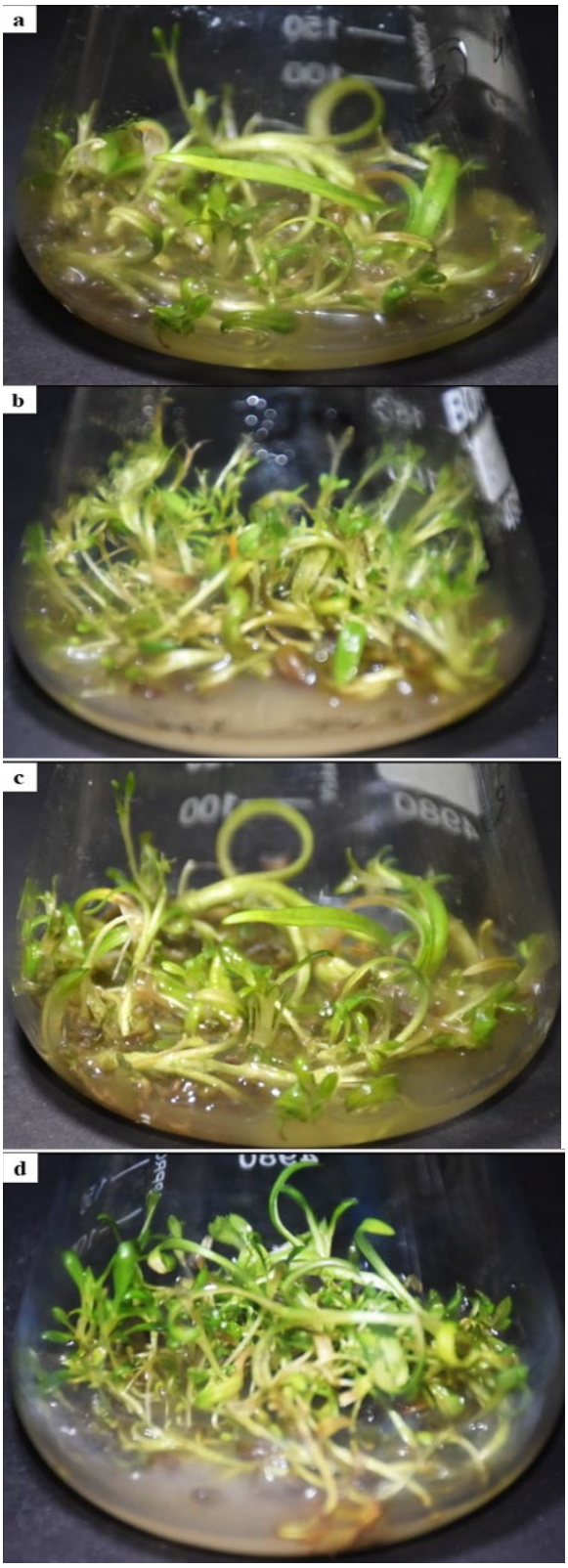

Figure 2. Elicitation of *G. kurroo* shoot cultures with various fungal extracts: (a). *Trichoderma viride,* (b). *Aspergillus niger,* (c). *Fusarium oxysporum,* (d). Endophytic Fungai isolated from *G. kurroo.*

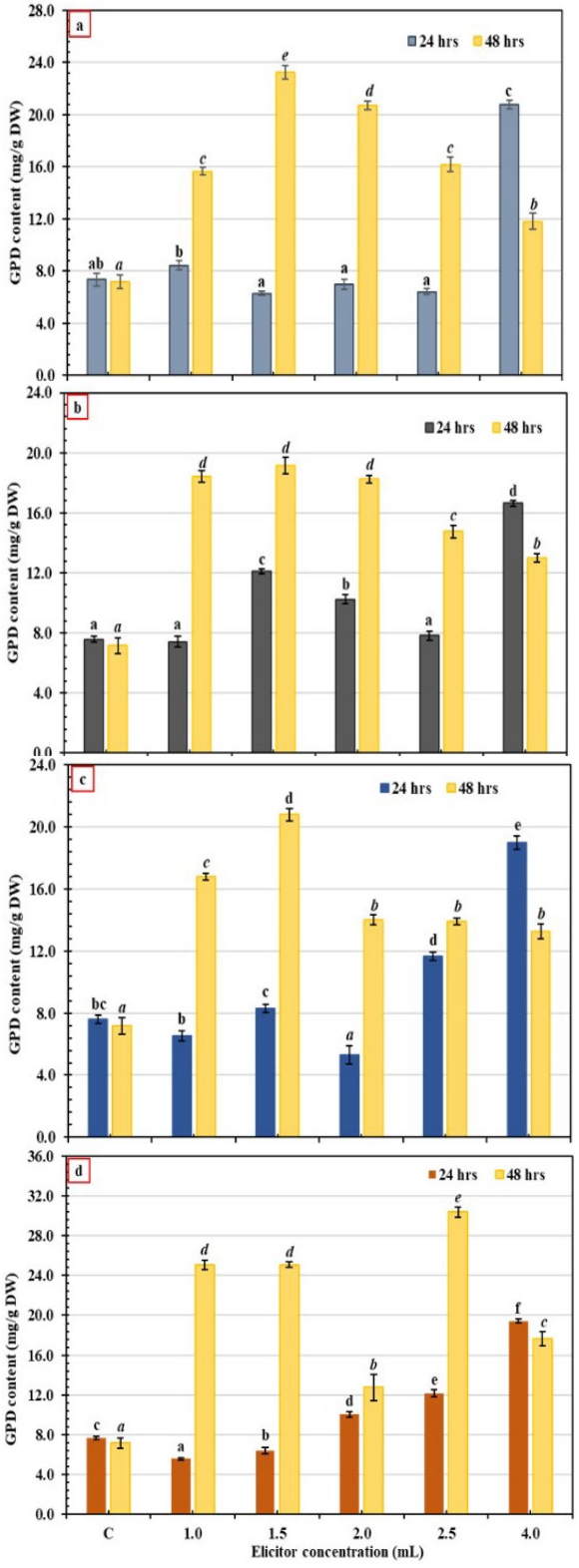

Figure 3. Effect of different fungal extracts on GPD synthesis: (a). *Trichoderma viride,* (b). *Aspergillus niger,* (c). *Fusarium oxysporum,* (d). Endophytic Fungai isolated from *G. kurroo.*

The highest amount of GPD accumulation was achieved with *P. album*, isolated from *G. kurroo* leaves compared to the other strains assessed. In addition, GPD accumulation varied based on the concentration and duration of elicitor treatments. This study provides a simple and efficient *in vitro* elicitation method for sustainable production of GPD using *G. kurroo*. Advancing this knowledge may conserve this important medicinal plant from extinction.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

ACKNOWLEDGEMENTS

The authors are grateful to the Vellore Institute of Technology management and the Dean SBST for their tireless support and encouragement. Our sincere thanks to BRNS, DAE – Govt. of India for providing financial support to A. Mariadoss - Senior Research Fellow – grant no. 35/14/42/2014-BRNS/2178.

ABBREVIATIONS

BLAST: Basic Local Alignment Search Tool
GPD: Gentiopicroside
IUCN: International Union for Conservation of Nature and Natural Resources
ITS: Internal Transcribed Spacer
PDA: Potato Dextrose Agar
HPLC: High-Performance Liquid Chromatography

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