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# COLCHICINE PRODUCTION IN *Gloriosa superba* CALLUSES BY FEEDING PRECURSORS

**G. Sivakumar,[1] K. V. Krishnamurthy,[2] J. Hao,[1] and K. Y. Paek[1]**

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*Phenylalanine and tyrosine play a central role in* Gloriosa superba *L. calluses for the production of colchicine. The lack of biosynthetic precursors and signal inducing enzyme activity are responsible for the lower production of colchicine in vitro. B5 medium nutrient grown calluses have a low content of colchicine, indicating that an optimal precursors level is required to increase PAL and TAL activity for colchicine accumulation. These results suggest that precursors are an important regulatory factor in colchicine accumulation in* in vitro.

**Key words**: Colchicine; HPLC-ESI-MS, phenylalanine, tyrosine.

Glory lily *(Gloriosa superba* L.) belongs to the family *Liliaceae*. It is an ancient medicinal plant in India. Indian was the first to use Ayurveda medicine, later as source of pharmaceutically important alkaloids such as colchicine and colchicoside [1]. Colchicine, the main alkaloid of *Gloriosa* species [2], was a useful agent in the treatment of acute attacks of gout [3]. Traditionally, colchicine was used for its antimitotic properties [4] and treatment of familial Mediterranean fever [5]. Colchicine has a well-known lipophilic drug action on tumors [6]. Derivatives of colchicine have shown promise as anticancer agents [7]. Recently, colchicine was used for gene expression and gene amplification [8].

*Gloriosa superba* callus culture may be an alternative source of phytocolchicine production. *In vitro* technology is essentially concerned with the application of the tools of natural product molecular science to the understanding of biological processes occurring in natural plant systems. This technology could be programmed with attempts to mimic some key parameters of biomolecular function exerted in the pharmaceutical molecules at a much simpler level. The main advantage of this technology is the year-round availability of plant material for colchicine production without geographical constraints; the products can be free of pesticide contamination, and production cost and time can be substantially reduced. At present, yields of the alkaloids are still too low to allow commercial application of callus cultures. The aromatic amino acids (phenylalanine, tyrosine, and tryptophan), which are derived from the shikimate pathway, are required as building blocks for protein synthesis. They are also required for the production of a large variety of secondary metabolites, including colchicine. Colchicine is a tricyclic alkaloid, the main features of which include a trimethoxyphenyl ring (A ring), a seven-membered ring (B ring) with an acetamide at the seven position, and a tropolonic ring (C ring). Phenylalanine and tyrosine are precursors of the colchicine alkaloids; phenylalanine ammonia lyase (PAL) and tyrosine ammonia lyase (TAL) are the first enzymes activated in this pathway, and they regulate the production of colchicine biosynthesis. PAL catalyzes the elimination of ammonia from *L*-phenylalanine to yield *trans*-cinnamic acid [9], and TAL catalyzes the formation of *trans-p*-hydroxycinnamic acid from *L*-tyrosine [10]. Here, we report the feeding of tryptophan, phenylalanine, and tyrosine, performed to increase the conversion of colchicine in *Gloriosa superba* callus culture.

1) Research Center for the Development of Advanced Horticultural Technology, Chungbuk National University, Cheongju 361-763, South Korea, fax +(8243) 275 74 67, e-mail: sivakumar@libero.it; b) Department of Plant Science, Bharathidasan University, Tiruchirappalli, India. Published in Khimiya Prirodnykh Soedinenii, No. 5, pp. 410-412, September-October, 2004. Original article submitted April 1, 2004.

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TABLE 1. Accumulation of Colchicine and Enzyme Activities in Precursor - Treated *G. superba* Calluses on the 25th Day

| Precursors (mM) | Colchicine content, % | PAL:TAL activites (mmol·mg protein$^{-1}$ h$^{-1}$) |
|---|---|---|
| Control (NAA 5.37) | 0.004[y] | 3.97±0.8:2.45±0.3[z] |
| Tryptophan (5) + NAA (5.37) | 0.02 d | 7.81±0.9:6.66±0.7 |
| Phenylalanine:tyrosine | | |
| 10:10 | 0.12 c | 7.96±0.4:7.99±0.2 |
| 20:20 | 0.16 b | 8.01±0.2:7.97±0.8 |
| 30:30 | 0.27 a | 8.94±0.2:8.91±0.3 |
| 40:40 | 0.07 e | 7.23±0.2:6.43±0.7 |
| 50:50 | 0.004 f | 4.14±0.7:4.02±0.2 |

[z]Mean ± standard error.

[y]Mean separation within columns by Duncan's multiple range test, $P \leq 0.05$.

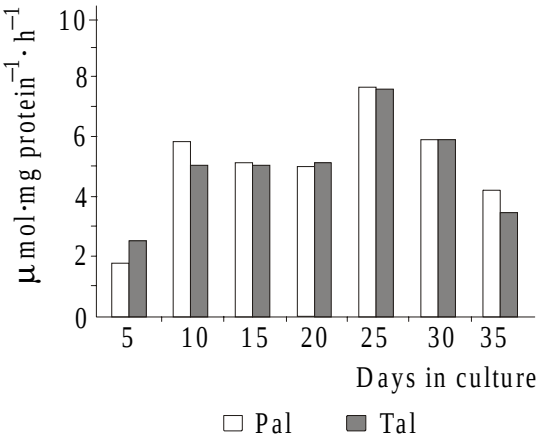


Fig. 1. Activities of PAL and TAL in precursors - treated calluses (phenylalanine at 30 mM and tyrosine at 30 mM).

Two procedural steps were followed for the enhanced colchicine content *in vitro*: the first step is the stimulation of calluses for primary metabolism using tryptophan, and the second step is the actual production of colchicine with the exogenous supply of phenylalanine and tyrosine. The calluses were induced from corm buds using $B_5$ medium supplemented with tryptophan in the range of 1–5 mM and NAA in the range of 1.35–5.37 mM. Maximum colchicine (0.02%) production was observed in the medium supplemented with tryptophan 5 mM and NAA 5.37 mM in 25 days. Colchicine production did not increase after 25 days. The activities of PAL and TAL were the maximum (7.81 mmol·mg protein$^{-1}$h$^{-1}$) during the 25th day and thereafter gradually decreased (data not shown).

When the concentrations of tryptophan and NAA were increased, the colchicine accumulation gradually decreased and the calluses became brown in color (data not shown). In the medium containing NAA alone without tryptophan, the calluses remained white in color and had low colchicine content (0.004%). If the callus proliferation time was prolonged, the colchicine accumulation decreased.

The above-mentioned medium-derived proliferating calluses were fed with both phenylalanine and tyrosine in the range of 10 mM to 50 mM. Calluses were subcultured in fresh medium with intervals of 7 days for 2–3 times. The maximum amount (0.27%) of colchicine was accumulated in the medium fed with 30 mM phenylalanine and 30 mM tyrosine after 25 days (Table 1). During this treatment the PAL and TAL activity reached maximum of 8.94 mmol·mg protein$^{-1}$ h$^{-1}$ (Fig. 1) and then sharply decreased after the 25th day. Increase in PAL and TAL activity was first detected on the 5th day, and maximum activity was attained on the 25th day, after which the activity gradually decreased. The maximal induction of PAL and TAL could play a role

in the conversion of colchicine intermediates. PAL and TAL activity in non precursor treated callus cells maintained relatively the same behavior but had low activity (data not shown).

The present study showed that the increasing colchicine content in precursor - treated callus might be due to the optimal increase in the endogeneous pool of *trans*-cinnamic acid and 4-hydroxycinnamate accounting for some part of the PAL and TAL response. Exogenous feeding of phenylalanine at 30 mM and tyrosine at 30 mM seemed to have a positive regulatory impact on the PAL and TAL activity in the callus, whereas higher concentrations were inhibited. The high endogeneous concentration of phenylalanine and tyrosine blocks the activation of PAL and TAL. It has been hypothesized that optimal concentrations of phenylalanine and tyrosine are required for the necessary concentration of *trans*-cinnamic acid and 4-hydroxycinnamate, along with a positive regulatory signal for colchicine biosynthesis.

In conclusion, regulation of precursor competence *in vitro* may be an increasing intermediate in the biosynthetic mechanism of alkaloid genes, which are responsible for the production of colchicine in calluses. It could be changed in the structure or expression of PAL and TAL regulatory signal transduction molecules. Further studies on differences in precursor levels must take into account the enhanced colchicine production of *Gloriosa superba* calluses.

## EXPERIMENTAL

**Fresh callus tissues** [11] were cultured on 100 ml of $B_5$ medium [12] supplemented with tryptophan, $\alpha$-naphthalene acetic acid (NAA), and 50 g/L sucrose. The medium i) contained no specific precursors of colchicine or ii) were fed with phenylalanine and tyrosine in the range of 10 mM to 50 mM concentrations for 25 days. All the cultures were incubated at 20°C and kept at a photoperiod of 16 h provided by cool, white fluorescent tubes with a photon flux density of 40 mmol $m^{-2}$ $s^{-1}$.

**The enzyme extraction method** has been described by Kavi Kishor [13]. PAL activity was assayed by following (*E*)-cinnamic acid formation at 280 nm in a spectrophotometer at 40°C in buffer (0.2 M sodium borate–NaOH, pH 8.7) containing 61 mM *L*-phenylalanine. TAL activity was assayed by monitoring *p*-coumaric acid formation at 310 nm in a spectrophotometer at 40°C in the same buffers containing 1.9 mM *L*-tyrosine according to the known method of Koukol and Conn [9]. Soluble proteins were estimated with bovine serum albumin as standard [14].

**A total alkaloid fraction** was generated by exhaustive extraction of freeze-dried callus by stirring the suspension for 12 h using cold methanol. The extraction was repeated with a further portion of extraction solvent until the extract remains colorless (100 g callus were extracted three times with 200 ml extraction solvent). The homogenate was centrifuged at 4000 rpm for 5 min as described by Finnie and van Staden [15]. The methanolic extract evaporated to dryness and redissolved in 50 ml $H_2O$ was then centrifuged for 5 min at 10.000 rpm. The supernatant was extracted twice with petroleum ether (bp 40–60°C) and washed once with diethyl ether and then five times with equal volumes of chloroform. After evaporation to dryness, the chloroform residue was redissolved in LC-MS CHROMASOLV® grade methanol and filtered through a 0.45 mm millipore filter.

The instrument used was an 1100 LC system (Hewlett-Packard, Waldbronn, Germany). A $C_{18}$ column ($250 \times 4.6$ mm) was used as the stationary phase. The mobile phase consisted of a mixture of methanol and 0.1% acetic acid solution (40:60) and the flow rate was 0.5 ml $min^{-1}$. The injection volume was 20 mL. The wavelength selected for UV detection was 254 nm. The entire column effluent was introduced directly into the electrospray ionization source without solvent splitting.

Accurate mass measurements and high-resolution mass spectra of colchicine were obtained using a Micromass, Quattro II triple quadrupole mass spectrometer (Germany). The system was monitored by a Philips Power PC computer equipped with the software's MassLynx for instrument control and data acquisition, data reprocessing, and solute quantification, respectively. The electrospray ion source in the MS was operated at 150°C in the positive ion mode. Nitrogen was used in both nebulizing and drying gas at a flow rate of 40 and 460 L/h, respectively.

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