Production of esculin by hairy root cultures of chicory
(Cichorium intybus L. cv. Lucknow local)

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Hairy root cultures established with Agrobacterium rhizogenes LMG 150 of an Indian cultivar of chicory produced esculin, a dihydroxycoumarin monoglucoside used as skin protectant and a marker in microbiological media. Maximum biomass of 6.2 ± 0.425 g/culture of transformed roots was obtained in full strength MS medium with 3% sucrose on 28th day. Esulin production was linked to growth and 100 g FW of transformed roots gave a maximum of 13.93 mg, corresponding to a 13-fold increase as compared to that in 30-day-old non-transformed roots (0.72 mg), during the same period.

Production of esculin has been reported in the leaves and bark of the horse chestnut tree, Aesculus hippocastanum1 and Fraxinus ornus bark2. This glucosyl coumarin finds importance for its skin protective properties and vitamin P activity3. The hydrolysis of esculin is an important marker in microbiological media for the detection of several bacteria including Clostridium thermocellum4, Yersinia enterocolitica5, Enterococci spp.6, Listeria spp.7, and for the differential isolation of certain fungi, like Aspergillus flavus from cotton seed8. Esulin was detected in potato tuber tissue having corky patch syndrome9, in decaying sweet potato roots10 and in wounded and deteriorated cassava roots11.

Esculin is also formed in the cell-free extract of Cichorium intybus flower heads from cichorin, an isomer of esculin by transglucosylation and not vice-versa12,13. As these reactions do not seem to proceed actively in the active tissues, esculin is found in meagre amounts in them. However, esculin is reported to be in relatively higher amount in the plant material during curing, followed by mixing of transglucosylase and vacuolar substrate14.

An investigation aimed at initiating hairy roots from an Indian cultivar of chicory (Cichorium intybus L. cv. Lucknow local) and a rapid method of esculin production from hairy roots is described in the present report. Another objective was to investigate the influence of different concentrations of MS medium with additional nitrate and phosphate supplementation, on the growth and esculin formation of hairy root cultures. Hairy root cultures have been developed as an alternate source for the production of root biomass and to obtain root derived compounds, these cultures has an additional advantage of higher growth rate and production of active principle in shorter period as compared to field grown plants, therefore it was of interest to develop hairy roots of chicory and to explore the possibility of production of esculin. This communication reports successful establishment of chicory hairy roots and study is oriented to enhancement of growth and production of esculin.

Materials and Methods

Bacterial strain—A. rhizogenes LMG 150, a mannopine-type strain was used in the present study. The bacteria were maintained on Agrobacterium Mannitol medium15 and subcultured every 28 days.

Fig. I—Esculin
Plant material—Seeds from ten different plants of same cultivar of *Cichorium intybus* Lucknow cv. local were obtained as gratis from the Bantha Research Station, National Botanical Research Institute (NBRI), Lucknow in the month of August, 1994.

Culture conditions—The seeds were washed in running tap-water and were surface sterilized by rinsing in 70% ethanol for 10 seconds followed by surface sterilization in aqueous solution of 0.1% (w/v) HgCl$_2$ for 3-4 min followed by 3-4 washes in sterile deionized water. The basal medium used was that of Murashige and Skoog$^{16}$ (MS) supplemented with 3% (w/v) sucrose. The pH of the medium was adjusted to 5.8±0.1 before gelling with 0.8% agar-agar (Himedia, India). The gelled medium was dispensed when warm, into 150×25 mm culture tubes (25 ml culture medium/tube), plugged with non-absorbent cotton and autoclaved at 1.06 kg cm$^{-2}$ pressure and 121°C for 15 min. The seeds were

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**Fig 2**—Growth of hairy root cultures of *C. intybus* in different media with following parameters analysed (a) Fresh weight (g/culture); (b) Dry weight (mg/culture); (c) Osmolarity (mOsmol/kg); (d) Conductivity (µS). In a culture period of 28 days. (Lines represent mean ±SD, n=3).
inoculated onto MS basal medium and incubated at 25°C±2°C under cool, white fluorescent light (4.4117 J m⁻² s⁻¹ 18 hr day⁻¹).

Establishment of hairy root culture—The hairy roots of C. intybus were induced by direct infection of shoot from 5 plantlets each 2 week old with A. rhizogenes LMG 150. Several hairy roots were found to arise from the inoculated site about 14±2 days after infection. The hairy roots emerging from the cut ends of the hypocotyl region were grown in MS basal medium. The hairy roots, five replicates each, were excised and placed on hormone-free MS liquid medium containing 0.5 mgL⁻¹ carbenicillin (HiMedia, Mumbai) to eliminate the bacteria. These were further cultivated in hormone-free MS liquid medium (30 ml/150 ml flask) containing 3% sucrose, double strength nitrate and phosphate, 1/2 strength MS and 1/4 strength MS. The cultures were kept on the rotary shaker at 110 rpm in dark. The roots were harvested after 4 weeks of culture and lyophilized (Hetofrig, UK).

Confirmation of hairy roots—Normal roots and hairy roots 10 g each FW were homogenised with a mortar and pestle, using acid washed and neutral sand, in 1 ml of extraction buffer¹⁷ comprising of 0.1 M Tris HCl, 0.5 M sucrose, 0.1% ascorbic acid, 0.1% cysteine HCl at pH 8. The slurry was centrifuged at 5000 rpm for 10 min. The supernatant concentrated to obtain 2 ml was used for the assay. Concentrates, 5 μL each, from the transformed and normal roots were spotted separately on Whatman #3 chromatographic paper along with standard mannopine (Sigma, Co. USA) on anodal side. The spotted samples were subjected to high voltage paper electrophoresis (1.5 KV) at 15 V cm⁻¹ for 30 min. in a buffer of formic acid, acetic acid and water (3:6:91 v/v/v) at pH 1.9. After drying overnight, the electrophoretogram was stained with silver nitrate¹⁸, later fixed with sodium thiosulphate solution (10% v/v in water) and washed afterwards in tap-water.

Measurement of growth parameters—Root growth and esculin content were examined in hairy roots cultured for 28 days in MS medium of full, half and quarter strengths, double nitrate and phosphate and compared with roots from 30-day old non-transformed plants. Growth of hairy roots of

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Fig. 3—HPLC profile of esculin eluted in pet. ether (60°-80°) with 5% ethyl acetate.

Fig. 4—Production of esculin (μg/culture) in hairy cultures of C. intybus grown in different types of media for a culture period of 28 days. (Lines represent mean ±SD, n=3).
C. intybus was determined by recording fresh and dry weights on respective days from the mean of three replicates. The fresh weights was measured after removing culture medium by filtration through a preweighed Whatman #1 filter paper under vacuum. The dry weight was estimated after the same sample was dried at 60°C for 48 hr. Conductometric estimation of the medium filtrate was carried out by using a conductivity meter (Wissenschafts-Werkstatten model LF-54, Wielhelm-Germany) and expressed in \( \mu \text{S} \) (microsiemen) units. Osmolarity was measured by using an automatic cryoscopic osmometer (Osmometer-030-Gonotech-Berlin, Germany) and expressed in \( \text{m Osmol kg}^{-1} \) units.

**Extraction and isolation of esculin**—The hairy roots (1500 mg) were lyophilised, powdered and exhaustively extracted at room temperature with eluent solvents in the order of increasing polarities from petroleum ether 60°-80°C, followed by increasing concentration of ethyl acetate up to 10%, chloroform, acetone and water. The extracts were separated into 6 fractions, each approx. 60 ml after filtering through Whatman #2 filter paper were concentrated to 2 ml. The fractions were subjected to HPLC along with standard esculin (Hi-Media, India) in a Shimadzu SCA-6A attached with SPD-6AV UV-VIS spectrophotodetector on an ODS column. The mobile phase was acetonitrile and water (80:20 v/v) and detector wavelength 313 nm; flow rate 1 ml min\(^{-1}\). The spectrofluorometric detection of esculin was further determined using the method of Puech and Moutounet.

**Results and Discussion**

The hairy roots of chicory could be commercially developed, so that it could serve as alternate source of food and pharmaceutical metabolites of interest. The hairy roots on MS medium were found to undergo active proliferation, negative geotropism and abundant lateral branching and were positive for mannopine. Chicory hairy roots attained a maximum biomass of 6.2±0.425 g /culture, corresponding to 0.8±0.06 g dry weight/culture, in 4 weeks (Fig. 2). While 30-day-old non-transformed roots yielded 0.72 mg esculin in 100 g FW of roots, the hairy roots were found to contain 13.93 mg esculin for the same fresh weight on a corresponding day. The HPLC analyses of normal and hairy roots showed formation of esculin (Fig. 3). The hairy roots cultured in full strength MS showed maximum esculin (209 \( \mu \text{g/culture} \) on 28th day of culture (Fig. 4). While MS medium with double phosphate showed maximum conductivity (9800 \( \mu \text{S} \)) on the zero day, the minimum conductivity on 28th day was seen in MS basal with double nitrate and 1/4th strength MS (120 \( \mu \text{S} \)). However, osmolarity being maximum in MS supplemented with double phosphate (340.8 m osmol/kg) on day zero, was found to be least in MS basal (26.5 m osmol/kg) on 28th day, indicating an inverse-correlation with growth rate in the latter culture system (Fig. 2). The effect of various treatments studied with reference to the growth of hairy roots and formation of esculin in C. intybus showed that decreasing the concentration of MS or doubling the nitrate or phosphate concentration in MS, had no effect in increasing the growth nor esculin content, beyond the labels obtained for MS, full strength medium.

Both osmolarity and conductivity in *Atropa*\(^2\), *Capsicum* and *Daucus*\(^2\) were found to decrease with increase in biomass in culture, thereby providing alternate methods for analyses of growth parameters. Medium conductivity and osmolarity depends on the electrolytic concentration and ignore the changes in sugars which is present in higher concentration than other media component put together as reported by Taya et al., both of these parameters which acts as an individual growth evidences, are in fact very sensitive to even show a smallest change in the biomass/ growth\(^2\).

The increase in esculin content in hairy roots is related to growth and increased lateral branching as compared to the normal roots, which is similar to other hairy root systems\(^2\). Hairy root cultures of chicory could be an alternative method to produce esculin. High-yielding hairy roots cultured in large quantities under aseptic conditions would enable in providing sufficiently high biomass with high esculin content. As plants show susceptibility to plant diseases and pests, this biotechnological approach to the industrial production of this dihydroxycoumarin glucoside would be expected to have significant advantages in producing this compound in a shorter period of time as compared to the traditional method of production which depends heavily on agroclimatic conditions.

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\textbf{References}