

**EFFICIENT REGENERATION AND HIGH ESCULIN CONTENT IN
CICHORIUM INTYBUS L., A POTENT MEDICINAL PLANT****ZAHID ALI¹, SHOWKAT. H. GANIE², ALKA NARULA¹, M. Z. ABDIN¹
AND P. S. SRIVASTAVA*¹**¹Department of Biotechnology, Hamdard University, New Delhi- 110062, India²Department of Botany, Hamdard University, New Delhi-110062, India**ABSTRACT**

In the present investigation, we have been able to establish a high regeneration protocol from leaves of *Cichorium intybus* L., a medicinally important herb. Maximum number of shoots (8.58 ± 0.31) with average length of 5.68 ± 0.20 cm was achieved on Murashige and Skoog (MS) medium fortified with 1.0 mg l^{-1} Kinetin (KN) and 0.5 mg l^{-1} Indole-3-acetic acid (IAA). Individual shoot implanted for rooting showed vigorous growth on MS medium supplemented with IAA (0.5 mg l^{-1}). Tissue culture raised plantlets were transferred for hardening in plastic pots containing soilrite and vermiculite (1:1). A total of 85.71% survival rate was recovered. Quantitative analysis of esculin content of *in vitro* and field grown plantlets was carried out by High Pressure Liquid Chromatography (HPLC). Esculin production was phenological stage specific, in 6 weeks regenerants esculin was $4.78 \pm 0.10 \text{ mg g}^{-1} \text{ dw}$, whereas $5.81 \pm 0.12 \text{ mg g}^{-1} \text{ dw}$ in *in vitro* flowering plants (10 weeks).

KEYWORDS Micropropagation, *Cichorium intybus* L, IAA, KN, Esculin**P. S. SRIVASTAVA**

Department of Biotechnology, Hamdard University, New Delhi- 110062, India

INTRODUCTION

Chicory (*C. intybus* L.) of Asteraceae was cultivated for the first time, as a medicinal plant in ancient Rome and Greece¹. Thereafter, it has been propagated throughout the world^{2, 3}. Different parts cure various ailments including allergy⁴, cancer⁵, aging⁶, bacterial activities⁷, virus⁸, inflammation⁹, herbicidal activity¹⁰ etc. In Indian traditional medicine (Ayurvedic and Unani) tonics have been prepared for the treatment of fever, diarrhoea, and enlargement of spleen¹¹. Decoction of leaves proves effective in the treatment of jaundice, liver enlargement, gout and rheumatism¹². A number of active principle has been isolated from whole or different plant parts which includes; inulin¹³, fructans, polyphenols, cichoric acid¹⁴, saccharides, organic acid, polyphenol¹⁵, alkaloid, triterpenes, sesquiterpenes, coumarins, flavones, esculin^{16, 17} etc which show many physiological bioactivities and therapeutic effects¹⁸. Esculin has been reported in the leaves^{19, 20} of *C. intybus*. Esculin though highly profitable for several protective effects^{21, 22}, it is present in a low concentration²³. Various factors (disease, pest, culture conditions, growth regulators, etc.) have been reported to be directly associated with growth and secondary metabolite production in plants^{24, 25, 26, 27, 28}. Although an important medicinal herb, *C. intybus* has not received the attention as it deserves. Multiplication of good number of propagules and improvement in the yield of esculin need sincere investigation. To produce true-to-type and disease free plants, *in vitro* culture is recognized as one of the key areas of biotechnology that has the potential to regenerate elites, conserve valuable plant genetic resources, obtain large quantity of plant material in relatively short period, better supply of propagules to the pharmaceutical companies and as an alternative means of secondary metabolite production^{29, 30}. Studies on the development of protocol for the clonal propagation of *C. intybus* have been reported but with low reproducibility and efficiency^{19, 31, 32, 33}. We have therefore, established a high regeneration protocol and quantified esculin production at different developing stages

(regenerants and *in vitro* flowering) and compared with field grown plant at flowering stage.

MATERIALS AND METHODS

(i) *Experimental material and surface sterilization*

Seeds of *C. intybus* procured in May 2009, from Herbal Garden at Jamia Hamdard, (Hamdard University), New Delhi, India. Seeds were surface sterilized with Streptomycin sulphate, 0.25 %, Bavistin, 0.5 % for 10 min and 0.1% HgCl₂ (Hi-media, India) for 10 min. Subsequently seeds were washed under aseptic conditions in laminar air hood by 70 % ethanol for 1 min and four times with sterile double distilled water.

(ii) *Shoot regeneration and multiplication*

Cotyledonary leaf, hypocotyls and leaf were used for shoot regeneration. Explants (1.2–1.5cm) were placed on MS³⁴ medium fortified with different concentrations of IAA, α -naphthalene acetic acid (NAA) and KN. Data was scored for percentage shoot regeneration, number of shoots and length.

(iii) *In vitro rooting*

After 6 weeks in regeneration medium, proliferating shoots (3.0 cm) were transferred to root induction medium (RIM). RIM comprised of different concentrations of IAA (0.25–2.0 mg l⁻¹). Data was scored after 3 weeks for % root induction, number and length.

(iv) *Ex-vitro transplantation*

Before transferring to plastic pots, rooted shoots were hardened for 3 weeks in half strength MS basal medium containing 0.5% (w/v) sucrose. Thereafter, plantlets with well-developed thick roots were transferred to pots containing equal amount of soilrite and vermiculite (1:1). Pots were covered with polyethylene bags to maintain 80–90% humidity and irrigated with tap water. Covers were withdrawn for 2–3 h every day for two weeks of five replicates.

(v) Culture conditions

The pH of the medium was adjusted to 5.6 and gelled with 0.8% (w/v) agar (Merck India Ltd., Mumbai) along with 3% sucrose (w/v). The medium was autoclaved at 121°C and 1.06 kg cm⁻² pressure for 20 min. The cultures were kept at 25.0 ± 2.0 °C under 16/8 hours (light /dark), photoperiod with white florescent light flux of 150-200 µE m⁻² s⁻¹ and relative humidity of 50-60.

(vi) HPLC Quantification of Esculin

In vitro and field grown samples (leaf) were dried at 40°C and powdered. 1 g of each sample was extracted with methanol in a Soxhlet. Solvent was concentrated till two ml was left. All the samples and standard were

filtered through 0.45µm pore size syringe before HPLC analysis was carried out. Reverse-phase HPLC (RP-HPLC) analysis was performed on Waters HPLC system (Binary Pump 600 controller), Waters PDA detector (996), Waters auto sampler (2707) and C₁₈ column (125×4 mm). Empower software was used for the data collection. RP-HPLC was performed with methanol: water (70:30), pH 3.5, at a flow rate of 1 ml min⁻¹ in isocratic mode. Esculin was quantified with the help of HPLC using different concentrations of standard esculin (M/S Sigma-Aldrich USA). Each extracts was injected in triplicate. Esculin content was expressed as mg gm⁻¹ dry weight basis.

RESULTS**Table 1**

Plant shoot regeneration from leaf explants cultured on MS medium containing different concentration of Indole-3-acetic acid (IAA) / α -naphthalene acetic acid (NAA) and Kinetin (KN) after six weeks.

Growth regulator (mg l ⁻¹)		Regeneration	Shoot formation	Length of shoot	Callusing
IAA	KN	(%)	(No. explant ⁻¹)	(cm)	(%)
0.0	0.0	00.00 ± 0.00	0.00±0.00	0.00±0.00	–
0.5	1.0	92.10 ± 3.51	8.58 ± 0.31	5.68±0.20	–
0.5	2.0	84.54 ± 3.20	7.10 ± 0.30	4.76±0.01	–
0.5	5.0	82.12 ± 1.13	5.20 ± 0.12	3.75±0.02	50.00±1.23
1.0	1.0	89.43 ± 2.42	7.40 8± 0.32	5.23±0.01	62.60±2.35
1.0	2.0	83.89 ± 3.90	6.54 ± 0.98	3.59±0.10	85.40±2.54
1.0	5.0	70.32 ± 2.30	4.42 ± 0.35	2.12±0.10	90.00±3.45
NAA		KN			
0.5	1.0	63.32 ± 3.30	5.80 ± 0.30	3.52±0.10	46.20± 2.32
0.5	2.0	50.23 ± 2.50	4.10 ± 0.30	4.95±0.02	65.00±2.67
0.5	5.0	43.21 ± 2.20	3.50 ± 0.20	4.20±0.20	83.60±3.19
1.0	1.0	72.76 ± 1.50	4.83 ± 0.20	3.50±0.20	85.00±2.65
1.0	2.0	50.67 ± 1.40	2.83 ± 0.30	3.06±0.10	89.00±3.22
1.0	5.0	41.55 ± 2.30	2.27 ± 0.30	2.54±0.40	95.00±2.73

-No callusing

Each value represents the mean ± standard error (S.E.) of twelve replicates per treatment in three repeated experiments.

(i) In vitro seed germination

The seeds initiated germination after 2-3 days of inoculation. Fully developed seedlings were obtained after 4 weeks.

Plant regeneration in *C.intybus* through leaf explants



Figure 1

Plant regeneration in *C.intybus* through leaf explants on MS (mg l^{-1}) + IAA (0.5) + KN (1.0). a Adventitious shoot buds emerging from the edge of explants. b Induction of multiple shoot buds from peripheral region of explants (four weeks). c Shoot multiplication (after six weeks of inoculation). d Plantlet with in vitro flowering (after ten weeks of inoculation). e Rooting in regenerated shoot on MS + IAA (0.5mg l^{-1}). f Well hardened plants of *C.intybus*.

(ii) Shoot regeneration and multiplication

Maximum regeneration was observed in leaf cultures on MS + IAA (0.5mg l^{-1}) + KN (1.0mg l^{-1}) supplemented medium followed by cotyledonary leaf and hypocotyl. The same medium also proved highly effective for shoot number and length. MS supplemented with

NAA and KN growth regulators did not prove as effective. NAA in place of IAA + KN in MS medium also induced maximum shoot regeneration and further multiplication, but the shoot length and number was maximum on NAA (0.5mg l^{-1}) + KN raised to 2.0mg l^{-1} (Table 1; Fig.1 a, b, c).

Table 2
Effect of different concentrations of Indole-3-acetic acid (IAA) on adventitious root formation from in vitro regenerated shoots

Auxin (mg/l)	Percentage response (Mean±SE)	No of roots (Mean±SE)	Root length(cm) (Mean±SE)
IAA			
0.0	65.0±2.10	6.40±0.20	2.30±0.05
0.25	81.5±3.40	9.30±0.17	2.80±0.01
0.50	100.0±3.20	16.50±0.32	3.60±0.03
1.00	94.21.0±2.60	13.00±0.23	3.20±0.02
2.00	87.7±1.80	7.80±0.18	2.50±0.01

Each value represents the mean ± standard error (S.E.) of twelve replicates per treatment in three repeated experiments.

(iii) Rooting

Individual shoots were placed for rooting on MS medium supplemented with different concentrations of IAA (0.0-2.0 mg l⁻¹). A linear increase in root induction percentage, number of root per shoot and root length was registered up to IAA (0.5 mg l⁻¹) in MS medium (Table 2; Fig.1 e). Cultures with increased concentration of IAA showed thin roots which became yellowish after three weeks.

(iv) Ex-vitro transplantation

After 3 weeks in half strength MS basal medium elongated, well developed rooted plantlets were gently removed from the culture tubes and transferred to plastic pots containing soil and vermiculite (1:1) (Fig.1 f). A total of 85.71% survival was noticed after 4 weeks.

Table 3
Esculin content in in vivo and in vitro samples

S.No.	Samples	Esculin content (mg g ⁻¹ dw)
1	<i>In vivo</i> (Flowering stage)	4.34 ± 0.64
2	Regenerants (six week)	4.78 ± 0.10
3	<i>In vitro</i> (Flowering stage)	5.81 ± 0.12

Each value represents the mean ± standard error (S.E.)

(vi) Quantification of Esculin

Quantitative estimation of esculin content was carried out in regenerants (Fig. 1c) and flowering plantlets (Fig. 1d). A comparison was made with field grown plants (flowering stage) 4.34 mg gm⁻¹dw. Maximum esculin was estimated from *in vitro* flowering plantlets (5.81 mg gm⁻¹ dw) followed by regenerants (4.78 mg gm⁻¹dw). Table 3 shows a comparative account of esculin production in *in vitro* and field grown plantlets.

DISCUSSION

Application of plant growth regulators plays a potent role in growth and development in plant systems. It is also responsible for secondary metabolite biosynthesis in plant cells and tissue cultures^{30, 35, 36}. In fact, type and ratio of

auxins and cytokinins have a dramatic role on both, growth and metabolite formation in plants³⁷. Auxin appears to be the primary factor controlling growth of cultured tissue and morphology of *in-vitro* developed roots, while the effects of cytokinins vary depending on secondary metabolite formation, as well as the relevant plant species^{37, 38}. In the present investigation we have used different explants (cotyledonary leaf, hypocotyle and leaf) of *in vitro* seedlings of *C. intybus* and established a high regeneration protocol. Leaf showed maximum regeneration and shoot multiplication. The combination of IAA with KN proved to be highly effective over NAA with KN. The results concur with the previous reports^{39, 40, 41, 42}. Maximum rooting was achieved on IAA fortified medium. IAA is known to be intimately involved in adventitious

roots formation^{43, 44} and also participates in controlling many phases of growth and differentiation^{45, 46}. IAA has been implicated in rooting in other plant systems as well⁴⁷. Hardening of rooted plantlets was also achieved. Such a hardening system has been successful in other plants as well^{26, 42, 47}. Plant cell and organ culture system offers viable alternative for the production of secondary metabolites that are of commercial importance in the food and pharmaceutical industries⁴⁸ and yield depends upon the cell proliferation and differentiation⁴⁹. Quantification of esculin content was carried out in *in vitro* grown plants and compared with field grown samples. In samples of *in vitro* plants, esculin content was higher than those of *in vivo* plants. Esculin production was age dependent. The results

are in agreement with the previous reports where a significant variation in secondary metabolites was noticed^{25, 30}.

CONCLUSION

An efficient high regeneration protocol has been developed from seedling leaf explants of *C. intybus in vitro* germinated seeds. The propagation protocol presented was found to be highly efficient and simple. This method can be beneficial for developing large scale multiplication systems for any propagation program for *C. intybus*. The high esculin content in micropropagated plants may help pharmaceutical industry.

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CONFLICT OF INTEREST: The authors declare that they have no conflict of interest.

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