Journal of Agricultural Technology

An efficient *in vitro* plant regeneration system for *Cichorium intybus* L.–an important medicinal plant

P. Velayutham[1*], B.D. Ranjithakumari[2], and P. Baskaran[2]

[1]Department of Botany, Government Arts College, Karur 639 001, India
[2]Department of Plant Science, Bharathidasan University, Tiruchirappalli 620 024, India

Velayutham, P., Ranjithakumari, B.D., and Baskaran, P. (2006). An efficient *in vitro* plant regeneration system for *Cichorium intybus* L.–an important medicinal plant. Journal of Agricultural Technology 2(2): 287-298

An efficient method for totipotent callus formation and whole plant regeneration has been developed for chicory (*Cichorium intybus* L.). Totipotent calli of chicory were induced from cotyledon, leaf, hypocotyl and root explants on MS medium supplemented with different concentrations of IAA, IBA, NAA and 2,4-D at 0.5-10 µM in combination with BAP (2 µM). These calli were transferred to shoot regeneration medium containing MS basal medium with different concentrations and combinations of BAP, KIN and IAA. Maximum number of shoots was obtained on MS medium with BAP (4 µM) + IAA (1 µM). The shoots were rooted on MS medium supplemented with IAA, IBA and NAA. Rooted plantlets were successfully established in the field after hardening.

Key words: chicory, callus, organogenesis, plant regeneration.

Abbreviations: BAP – 6-benzylaminopurine; 2,4-D – 2,4-dichlorophenoxyacetic acid; IAA – indoleacetic acid; IBA – indolebutric acid; KIN – kinetin (6-furfuryl aminopurine); NAA – naphthaleneacetic acid; PGRs – plant growth regulators.

Introduction

Chicory (*Cichorium intybus* L.), a member of *Asteraceae*, is a biennial plant, and is one of the important medicinal plants cultivated throughout India. The whole plant (root, leaf, and seed) is used medicinally, because of the presence of inulin, a major reserve carbohydrate, bitter sesquiterpene lactones, coumarins, flavonoids and vitamins. It is used as anti-hepatotoxic, anti-ulcerogenic, anti-inflammatory, appetiser, digestive, stomachic, liver tonic, cholagogue, cardiotonic, depurative, diuretic, emmenagogue, febrifuge, alexeteric and tonic. The chicory plant is used for treating AIDS, cancer, diabetes, dismenorrhoea, impotence, insomnia, splenitis, and tachicardia

[*]Corresponding author: P. Velayutham; e-mail: vela_utham@yahoo.com

(Duke, 1983). Research has assessed the anti-fungal (Monde *et al*., 1990), postcoital contraceptive (Keshri *et al*., 1998), anti-ulcerogenic (Ahmad *et al*., 1998), anti-inflammatory (Ki *et al*., 1999), anti-hepatotoxic (Gadgoli and Mishra, 1997; Mitra *et al*., 1998) and anti-cancer (Hughes and Rowland, 2001) properties in *C. intybus*. Due to the non-digestibility of inulin, a major reserve food in chicory, it is suitable for consumption by diabetics (Niness, 1999) and is used in inulin clearance test to measure glomerular filtration rate – GFR (Vasudevan and Sreekumari, 2002). The roots, leaves, flowers and chicons are used in cuisine in the form of salads, pickles and sauces. The root is rich in alkaloids which forms an ingredient or adulterant to replace coffee (Jung *et al*., 1996). The deep purple flower heads yield blue dye (Bremness, 1988).

Chicory contains a variety of bioactive compounds, viz., inulin, sesquiterpene lactones and coumarins, which play a key role in medicinal and dietary purposes. Among coumarins, esculin, esculetin, cichoriin and umbeliferone have been reported in chicory (Evans, 1996; Bais *et al*., 1999, 2000, 2001; Rehman *et al*., 2003).

Organogenesis is a suitable method for obtaining a large quantity of genetically homogenous and healthy plant material, which can be used for planting (Pierik, 1987). In order to diffuse cultivation of medicinal plants, the first step is the production of high quantities of healthy and genetically homogeneous plant material, which can be propagated at low cost.

Though the conventional breeding techniques have considerably increased the productivity of modern crops, the application of biotechnology could speed up further crop improvement. It overcomes the barriers in conventional vegetative propagation and fulfils the demand for large-scale cultivation in a short period by rapid mass multiplication. To date we can speed up the production rate of the average plant by approximately 10,000 times and a large number of productive plants can be multiplied routinely through tissue culture (Rao *et al*., 1996). The aim of our present study is to enhance the competence and swiftness of shoot regeneration from leaf, cotyledon, hypocotyl and root tissues of *C. intybus* and this protocol is effectively used in *Agrobacterium* mediated gene transformation for crop improvement.

Materials and methods

Plant material

Field grown plants were used as source of explants. Cotyledon, leaf, hypocotyl and root of seven day old seedlings were selected as explants for indirect regeneration. The explants were washed in running tap water for 30 minutes. Then they were washed in an agitated solution of liquid detergent 2%

(v/v) (Teepol) for two minutes and rinsed in distilled water three times. Surface sterilization was performed by immersion of the explants in 70% (v/v) aqueous ethanol for 40 seconds followed by 0.1% (w/v) mercuric chloride for five minutes. Finally, the materials were thoroughly rinsed with sterile distilled water five times to remove the traces of mercuric chloride. All the explants were cut into pieces approximately 10–15 mm long for inoculation.

Culture conditions

The media used were based on MS formulation (Murashige and Skoog, 1962), containing 3% (w/v) sucrose (Himedia, India), 0.8% (w/v) agar (HiMedia, India). The pH of the medium was adjusted to 5.8 before autoclaving for 15 minutes at 121ºC. The explants, viz., cotyledon, hypocotyl, leaf and roots were placed on culture tubes (150 × 25 mm) containing 10-15 ml of medium with respective growth regulators. The cultures were incubated at 25±2ºC under 16/8 hours (light/dark) photoperiod with white fluorescent light giving a photon flux density of 150-200 $\mu Em^{-2} s^{-1}$ and relative humidity of 50-60%.

Callus induction

The explants were cultured in MS medium supplemented with various concentrations and combinations of plant growth regulators including 0.5, 2.5, 5, 7.5, and 10 µM of IAA, IBA, NAA or 2, 4-D in combination with 2 µM BAP.

Shoot regeneration

Callus was cultured on MS medium supplemented with various concentration and combinations of plant growth regulators including: 2–10 µM BAP or Kin and 0.5 -2.5 µM IAA.

Root induction

The shoots were excised from regeneration medium and were transferred to MS medium supplemented with different concentrations of IAA, IBA and NAA at 2.5, 5.0, 7.5, 10.0, and 12.5 µM.

Acclimatization

Rooted plantlets were gently washed with running tap water and followed by sterile distilled water and then successfully transferred to pots containing sterile garden soil. The pots were covered with porous polyethylene bags for maintaining high humidity. The hardened plants were maintained in the culture room. The polyethylene bags were removed after 2 weeks and then the hardened plantlets were successfully transferred to normal room temperature. These plants were lucratively transferred to net house for further growth and development.

Statistical analysis

Experiments were set up in a Randomized Block Design (RBD) and each experiment was replicated thrice. Observations were recorded on the percentage of response, number of shoots per explant, number of roots per shoot and root length. Means and standard errors were carried out for each treatment.

Results and discussion

Effect of growth regulators on callus induction

In the present study, different types of explants (cotyledon, leaf, hypocotyls and root) were treated with different concentrations and combinations of plant growth regulators for plant regeneration. Similarly, various explants were used for shoot regeneration in *C. intybus* (Vermeulen *et al*., 1993; Margara and Rancillac, 1996; Wagner and Eneva, 1996). Among the four auxins tested, NAA, IBA and IAA were found to be more effective in callus induction than 2, 4-D (Table 1). The order of effectiveness of plant growth regulators for callus induction was NAA>IBA>IAA>2, 4-D. Similar effects were obtained by several authors (Wagner and Gailing, 1996; Rehman *et al*., 2003). However, green compact callus was developed on MS medium supplemented with 0.5–10 µM IAA, IBA, NAA, 2, 4-D in combination with 2 µM BAP in cotyledon, hypocotyl, leaf and root explants after two weeks of culture. The order of effectiveness was leaf>cotyledon>hypocotyl>root. Similar results in chicory and other plants are also well documented (Eung *et al*., 1999; Rehman *et al*., 2003).

Table 1. Effect of PGRs on callus induction in *Cichorium intybus* L.

Concentrattions of PGRs (µM)	Cotyledon	Leaf	Hypocotyl	Root
0.5 IAA + 2.0 BAP	43.00±1.13	46.17±1.96	35.00±2.35	31.67±1.36
2.5 IAA + 2.0 BAP	82.83±0.96	80.00±4.08	51.67±1.36	43.33±1.96
5.0 IAA + 2.0 BAP	90.33±0.98	86.67±1.36	66.67±1.36	47.67±1.18
7.5 IAA + 2.0 BAP	87.33±1.18	87.00±1.24	66.67±2.72	53.37±1.36
10.0 IAA + 2.0 BAP	82.67±1.14	82.67±1.67	61.67±1.36	52.67±2.17
0.5 IBA + 2.0 BAP	43.33±1.36	43.33±2.72	36.67±3.60	33.33±1.36
2.5 IBA + 2.0 BAP	66.67±2.72	76.67±2.72	61.67±1.36	36.67±3.60
5.0 IBA + 2.0 BAP	91.67±1.36	93.33±1.76	71.67±5.93	48.33±3.60
7.5 IBA + 2.0 BAP	90.00±2.35	91.67±2.72	721.67±3.60	61.67±1.36
10.0 IBA + 2.0 BAP	86.67±1.36	88.33±1.36	63.33±1.36	60.00±2.35
0.5 NAA + 2.0 BAP	33.33±1.36	45.00±2.35	26.67±3.60	23.33±2.72
2.5 NAA + 2.0 BAP	73.33±1.36	68.33±3.60	53.33±4.90	33.33±2.72
5.0 NAA + 2.0 BAP	86.67±3.60	91.67±2.72	68.33±2.72	53.33±7.20
7.5 NAA + 2.0 BAP	93.33±1.36	96.67±1.36	63.63±1.36	53.33±5.44
10.0 NAA + 2.0 BAP	91.67±1.36	91.67±1.36	63.33±3.60	48.33±1.36
0.5 2,4-D + 2.0 BAP	8.33±1.36	6.67±1.36	--- ---	
2.5 2,4-D + 2.0 BAP	53.33±4.90	45.00±6.23	38.33±4.90	26.67±3.60
5.0 2,4-D + 2.0 BAP	88.33±3.60	83.33±1.36	53.33±3.60	36.67±4.90
7.5 2,4-D + 2.0 BAP	81.67±3.60	83.33±3.60	55.00±2.33	38.33±3.60
10.0 2,4-D + 2.0 BAP	86.67±4.90	83.33±1.36	60.00±2.35	36.67±2.72

Values are Mean±SE of ten replicates per treatment and repeated three times.
The data were recorded after 30 days culture.

Higher frequency of callus was observed on MS medium supplemented with NAA and IBA in combination with 2 µM BAP after 30 days of culture. The combination of auxins with cytokinins was effective for prospective callus. A similar result was observed by Rehman *et al*. (2003). The optimum callus was obtained from 7.5 µM NAA + 2 µM BAP in leaf (Fig. 1A) and cotyledon

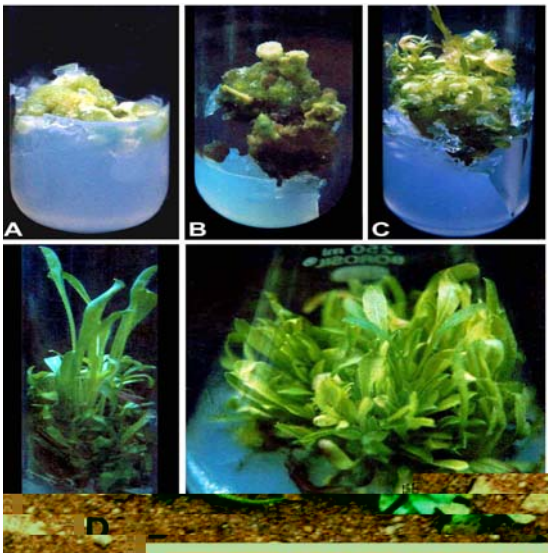

Fig.1. *In vitro* regeneration of *Cichorium intybus* L. **A.** Callus induction on MS + 7.5 µM NAA + 2.0 µM BAP in leaf explant. **B.** Green compact callus of leaf explant. **C.** Shoot bud regeneration from green compact callus.**D.** Shoot elongation in MS + 4.0 µM BAP + 0.3 µM Kin.**E.** Shoot elongation in MS + 4.0 µM BAP + 1.0 µM IAA.

explants; 7.5 µM IBA + 2 µM BAP in hypocotyl and root explants (Table 1). Similarly, BAP and NAA combination was found to induce more shoots in *Syzygium aromaticum* (Mathew and Hariharan, 1990). In contrast to our results, IAA and BAP combination induced callus under darkness in *Hypericum erectum* (Yazaki and Okuda, 1990). In each combination, the nature of the callus (white friable, green friable, green compact and whitish-yellow friable) varied. Leaf and cotyledon calli were subcultured in MS medium containing 7.5 µM NAA + 2 µM BAP whereas hypocotyl and root calli in 7.5 µM IBA + 2 µM BAP. The subcultured calli enhanced shoot regeneration potential after two weeks.

Callus induction and shoot regeneration system are known to be very useful for the study of biosynthesis of natural products and the factors that influence them, giving some possibilities of controlled production. In several plant species, this approach has been applied (Smollny *et al*., 1992; Schimdt *et al*., 2000).

Shoot regeneration

Cotyledon, hypocotyl, leaf and root explants derived calli were cultured on MS medium supplemented with various concentrations and combinations of

Table 2. Effect of various PGRs on shoot regeneration from callus of *Cichorium intybus* L.

Concentrations of PGRs (µM)			**Percentage of**	**Number of**
BAP	**KIN**	**IAA**	**Response**	**Shoots**
2.0	--	--	75.00±6.23	7.14±1.85
4.0	--	--	100.00±0.00	12.57±2.44
6.0	--	--	99.16±1.07	12.28±2.06
8.0	--	--	85.00±4.08	10.00±2.68
10.0	--	--	81.67±3.60	8.00±0.83
--	2.0	--	56.67±2.72	2.00±0.34
--	4.0	--	76.67±3.60	3.62±0.90
--	6.0	--	75.00±2.35	4.40±0.80
--	8.0	--	63.33±2.72	5.00±1.17
--	10.0	--	71.67±3.60	3.28±0.43
4.0	1.0	--	85.00±2.35	10.42±1.77
4.0	2.0	--	91.67±1.36	13.71±2.11
4.0	3.0	--	83.33±3.60	16.42±4.27
4.0	4.0	--	86.67±1.36	10.85±1.78
4.0	5.0	--	83.33±1.36	8.85±1.99
4.0	--	0.5	95.00±2.35	30.43±1.83
4.0	--	1.0	100.00±0.00	42.92±0.89
4.0	--	1.5	90.00±0.00	34.26±2.74
4.0	--	2.0	86.67±2.55	21.27±1.76
4.0	--	2.5	86.67±1.36	15.48±2.49

Values are Mean±SE of ten replicates per treatment and repeated three times.
The data were recorded after 8 week culture.

Table 3. Effect of different auxins on root induction from regenerated shoots of *Cichorium intybus* L.

Concentrations of Auxins (μM)	Percentage of Response	Number of Roots	Root Length (mm)
2.5 IAA	46.67±1.36	13.67±3.05	61.33±4.85
5.0 IAA	93.33±1.36	12.33±3.21	53.26±2.62
7.5 IAA	80.00±2.35	29.33±1.52	59.87±1.73
10.0 IAA	66.67±3.60	36.67±3.51	43.97±5.55
12.5 IAA	40.00±2.35	34.00±5.29	46.77±2.08
2.5 IBA	60.00±8.16	7.67±4.72	69.12±3.03
5.0 IBA	78.33±3.60	16.67±2.51	68.47±3.58
7.5 IBA	86.67±1.36	13.67±2.08	53.26±2.62
10.0 IBA	96.67±2.72	41.33±6.04	29.50±3.87
12.5 IBA	26.67±9.81	65.33±5.51	27.89±2.17
2.5 NAA	23.33±2.72	5.33±1.78	60.47±4.85
5.0 NAA	93.33±2.72	14.67±1.48	56.00±3.67
7.5 NAA	85.00±2.35	46.67±4.51	60.05±3.78
10.0 NAA	56.67±7.20	40.33±3.12	28.02±2.05
12.5 NAA	16.67±2.72	42.87±3.23	29.62±1.34

Values are Mean±SE of ten replicates per treatment and repeated three times.
The data were recorded after 30 days culture.

Acknowledgements

The first author is thankful to Dr. P. Jayaraman, Plant Anatomy Research Centre, Chennai and Dr. T. Bhupathi, Reader, Government Arts College, Karur for giving decisive and valuable suggestions.

References

Agretious, T.K., Martin, K. and Hariharan, M. (1996). *In vitro* clonal multiplication of *Alpinia calcarata* Rosc. Phytomorphology 46: 133-138.

Ahmad, K.D., Gilani, S.N., Akhtar, A.H. and Khan, L. (1998). Antiulcerogenic evaluation of aqueous extracts of *Cichorium intybus* and *Phyllanthus emblica* in normal and aspirin-treated rats, Pakistan. Journal of Science and Industrial Research 41: 92-96.

Anand, P.H.M., Harikrishnan, K.N., Martin, K.P. and Hariharan, M. (1997). *In vitro* propagation of *Kaempferia rotunda* Linn. "Indian crocus" - A medicinal plant. Phytomorphology 47: 281-286.

Bais, H.P., George, J. and Ravishankar, G.A. (1999). Influence of polyamines on growth of hairy root cultures of witloof chicory (*Cichorium intybus* L. cv. Lucknow local) and formation of coumarins. Plant Growth Regulation 18: 33-37.

Bremness, L. (1988). The Complete Book of Herbs: Joanna Chisholm. Darling Kindersley, London.

Duke, J.A. (1983). Medicinal Plants of the Bible: Out of print Trado-Medic Books. Buffalo, NY.

Eung, J.P., Lim, H.T., Part, E.J., Lim, H.T., and Lee, J.M. (1999). Establishment of an efficient *in vitro* plant regeneration system in chicory (*Cichorium intybus* L. var. *sativus*). Acta-Horticulture 483: 367-370.

Evans, W.C., (1996). Ed. Trease and Evans' Pharmacognosy. 14th Edition., W.B. Saunders Company Limited, London.

Gadgoli, C. and Mishra, S.H. (1997). Antihepatotoxic activity of *Cichorium intybus*. Ethanopharmacology 52: 131-134.

Hughes, R. and Rowland, I.R. (2001). Stimulation of apoptosis by two prebiotic Chicory fructans in the rat colon. Carcinogenesis 22: 43-47.

Jung, G.A., Shaffer, J.A., Varga, G.A. and Everhart, J.R. (1996). Performance of 'Grassland Puna' chicory at different management levels. Agronomy 88: 104-111.

Keshri, G., Lakshmi, V. and Singh, M.M. (1998). Postcoital contraceptive activity of some indigenous plants in rats. Contraception (Stoneham) 57: 357-360.

Ki, C.G., Yim, D. and Lee, S.Y. (1999). Biological activities of root of *Cichorium intybus*. Nat. Prod. Science 5: 155-158.

Manickam, R., Elango Mathavan, R. and Antonisamy, R. (2000). Regeneration of Indian ginseng plantlets from stem callus. Plant Cell Tissue and Organ Culture 62: 181-185.

Margara, J. and Rancillac, M. (1996). Recherches experimentales sur la neoformation de bourgeons inflorescentiels on vegetatifs *In vitro* a partir d'explantats d'endive (*Cichorium intybus* L.), II. Observations sur la vernalisation prealable de la racine. Annales de physiologie vegetale 8: 39-47.

Mathew, M.S. and Hariharan, M. (1990). *In vitro* multiple shoot regeneration in *Syzygium aromaticum*. Annals of Botany 65: 277-279.

Mitra, S.K., Venkataranganna, M.V, Sundaram, R. and Gopumadhavan, S. (1998). Protective effect of HD-03, a herbal formulation, against various hepatotoxic agents in rats. Ethnopharmacology 63: 181-186.

Monde, K.O., Shira, A. and Takasugi, M. (1990). A guaianolids phytoalexin, cichorlexin, from *Cichorium intybus*. Phytochemistry 29: 3449-3451.

Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassay with tobacco tissue culture. Physiologia Plantarum 15: 473-497.

Nikam, T.D. and Shitole, M.G. (1993). Regeneration of niger (*Guizotia abyssinica* cass.)cv Sahyadri from seedling explants. Plant Cell Tissue and Organ Culture 32: 345-349.

Niness, K.R. (1999). Inulin and Oligofructose. Nutrition 129: 1402S-1406S.

Pierik, R.L.M. (1987). Ed. *In vitro Culture of Higher Plants*. Martinus Nijhoff Publishers. The Hague.

Purahit, S.D., Dave, A. and Kukda, G. (1994). Micropropagation of safed musli (*Chlorophytum borivilianum*) a rare medicinal herb. Plant Cell Tissue and Organ Culture 39: 93-96.

Rao, P.S., Supresanna, P. and Ganapathi, T. (1996). Plant biotechnology and Agriculture: Prospects for crop improvement and increasing productivity. Science and Culture 62: 185-191.

Regman, R.W., Israr, M., Srivastava, P.S., Bansal, K.C. and Abdiv M.Z. (2003). *In vitro* regeneration of witloof chicory from leaf explants and acculmulation of esculin. In Vitro Cellular Developmental Biology-Plant 69: 442-446.

Rehman, R.U., Israr, M., Srivastava, P.S., Bansal, K.C. and Abdin, M.Z. (2003). *In vitro* regeneration of witloof chicory (*Cichorium intybus* L.) from leaf explants and accumulation of esculin. In vitro Cellular Developmental Biology Plant 39: 142-146.

Schimdt, W., Peters, S. and Beerhues, L. (2000). Xanthone 6-hydroxylase from cell cultures of *Centaurium erythraea* and *Hypericus androsaemum* L.. Phytochemistry 53: 427-433.

Smollny, T., Wichers, H., de Rijk, T., van Zwan, A., Shasavari, A. and Alfermann, W. (1992). Formation of lignans in suspension cultures of *Linum album*. Planta Medica 58: 622-624.

Sudha, C.C. and Seeni, S. (1994). *In vitro* propagation and field establishment *Adhatoda beddomei* CB Clarke: a rare medicinal plant. Plant Cell Reporter 13: 203-207.

Tiwari, K.N., Sharma, N.C., Tiwari, V. Singh, B.D. (2000). Micropropagation of *Centella asiatica* (L.) a valuable medicinal herb. Plant Cell Tissue and Organ Culture 63: 179-183.

Vasudevan, D.M., and Sreekumari, S. (2002) Ed. Textbook of Biochemistry for Medical Students. 3rd edition, Jaypee Brothers, New Delhi.

Vermeulen, A., Desprezy, B., Lenoir, P., Chupeau, M.C. and Chupeau, Y. (1993). Isolation and culture of mesophyll protoplasts from two cytoplasmic albino mutants of *Cichorium intybus* L. Plant Physiology 142: 377-380.

Wagner, G.M. and Eneva, T. (1996). Positive effect of cefotaxime on plant regeneration from *Cichorium intybus* L. leaf material. Landbauforschung Volkenrode 46: 166-168.

Wagner, G.M. and Gailing, O. (1996). The influence of different carbon sources on callus formation and shoot proliferation of chicory (*Cichorium intybus* L. var. *sativum*). Landbauforschung Volkenrode 46: 1-4.

Yazaki, T. and Okuda, T. (1990). Procyaninins in callus and multiple shoots of *Hypericum erectum*. Planta Medica 56: 490-491.

Yu, T.A., Yeh, S.D., Cheng, Y.H. and Yang, J.S. (2000). Efficient rooting for establishment of papaya plantlets by micropropagation. Plant Cell Tissue and Organ Culture 61: 29-35.

(Received 21 July 2006; accepted 17 October 2006)