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

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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

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(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

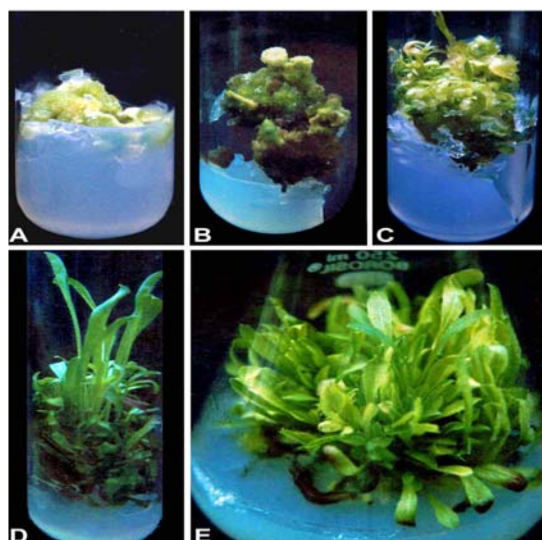

Fig.1. *In vitro* regeneration of *Cichorium intybus* L. **A.** Callus induction on MS + 7.5 µM NAA + 2.0 µM BAP in leaf explant. **B.** Green compact callus of leaf explant. **C.** Shoot bud regeneration from green compact callus.**D.** Shoot elongation in MS + 4.0 µM BAP + 0.3 µM Kin.**E.** Shoot elongation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

BAP and/or KIN and IAA (shoot regeneration medium). In this study, shoot induction was found to be greater in BAP. Similar results were well documented in *Niger* (Nikam and Shitole, 1993) and *Chlorophytum* (Purahit *et al*., 1994). Calli turned green compact (Fig. 1B) and shoot buds were induced after four weeks of culture (Fig. 1C). All of the explants derived calli were not assorted in shoot bud regeneration (data not shown). Higher frequency of shoot regeneration was obtained in MS medium supplemented with 4 µM BAP, 6 µM Kin, 4 µM BAP + 0.3 µM Kin and 4 µM BAP + 1.0 µM IAA (Fig 1D; Table 2).

The observation on the synergistic effect of BAP in combination with IAA on promotion of shoot regeneration of this medicinal plant species is in agreement with that of several plant species (Sudha and Seeni, 1994; Manickam *et al*., 2000). Conversely, Kin, IAA and CH proved best response in shoot regeneration in witloof chicory (Regman *et al*., 2003). However, maximum number of shoots were obtained in MS medium fortified with 4 µM BAP + 1 µM IAA (Table 2; Fig. 1E). The beneficial (4–5 cm long) shoot length was observed in all the concentration and combinations of shoot regeneration medium after 8 weeks culture.

Effect of auxins on rooting of shoots

Elongated shoots were excised from each regeneration medium and then transferred to MS medium supplemented with 2.5–12.5 µM IAA, IBA or NAA (Table 3). However, IBA was found to be the best rooting hormone than IAA or NAA. Similar results are well documented (Tiwari *et al*., 2000; Yu *et al*., 2000; Rehman *et al*., 2003). Higher frequency (96.6%) of root induction was obtained in MS medium supplemented with 10 µM IBA. The maximum number of roots was observed in 12.5 µM IBA (Fig. 2A). However, the root length was varied in each treatment (Table 3). MS medium supplemented with IBA (10 µM) was produced thick and hair-like secondary roots. Roots in IAA and NAA media were thin and abundant hair-like secondary roots. While rooting, the flower heads were also developed and opened in a long photoperiod (Figs 2A, B).

Acclimatization and establishment of soil

The rooted plantlets were carefully taken from the culture tubes and then washed with tap water and followed by sterile water. After washing the rooted plantlets were successfully transferred to pots containing sterilized garden soil with 100% survival (Fig. 2C). Sterilized garden soil minimized the cost of transplantation as documented by several authors (Agretious *et al*., 1996;

Anand *et al*., 1997). The acclimatized plantlets were successfully established in the field (Fig. 2D)

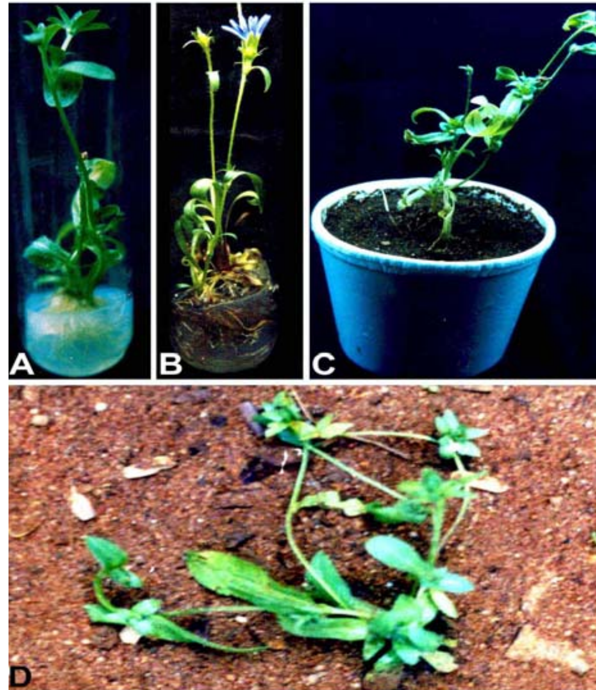

Fig 2. *In vitro* regeneration of *Cichorium intybus* L. **A.** Shoot with rooting in MS + 12.5 µM IBA.**B.** Rooted plantlet with opened flower heads. **C.** Hardened plant in pot. **D.** *In vitro* derived plant established in the field.

Conclusions

The present study has resulted in the establishment of protocol for indirect organogenesis of *Cichorium intybus* through leaf, cotyledon, hypocotyl and root explant derived callus. In this study, leaf explant derived callus produced a higher number of shoots. This technique could be used as a tool to introduce new variants of *C. intybus* in a somaclonal variant selection programme.

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.

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