Original Article

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High-frequency plant regeneration and histological analysis of callus in *Cichorium intybus*: An zimportant medicinal plant

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ABSTRACT

An efficient *in vitro* propagation and *in vitro* flowering protocols were developed for the medicinal plant *Cichorium intybus* (Asteraceae) using leaf disc explants. Media supplemented with the growth regulator naphthalene acetic acid (NAA) (1.5 mg/l) + 6-benzyle adenine (0.25 mg/l) was used for the initial induction of the callus and further subcultured to the same media for the proliferation of the callus. Pale yellow and green calli were noticed, which depends on incorporation of the growth hormones and their varying concentrations. Murashige and Skoog medium in addition with 2 mg/l kinetin+ 0.5 mg/l indole-3-acetic acid (IBA) + 500 mg/l casein hydrolysate resulted in maximum regeneration. Media supplemented via IBA (0.5 mg/l) and NAA (0.5 mg/l) (98%) was found to be optimum for rhizogenesis for *in vitro* regenerated plants. For acclimatization 5-6 weeks mature in *vitro* regenerated plants were transferred into the greenhouse for acclimatization. The histological study revealed the presence actively dividing meristematic cells in callus. The occurrence of the peripheral meristematic zone associated with callus was noticed in after 20 days, which formed the shoot meristems after 45 days of incubation. To our knowledge, this is the first report on high-frequency plant regeneration which was carried out indirectly from the leaf explants which was grown in controlled environment with varying concentration of the growth regulators and histology of callus of different stages from leaf explants of *C. intybus.*

KEY WORDS: *Cichorium intybus* (chicory), high-frequency plant regeneration, histological studies, leaf explants

INTRODUCTION

Cichorium intybus (Chicory) member of *Asteraceae* family. It is a biennial herb consisting of the tuberous tap root. It is an important medicinal herb used in Ayurveda, Unani, and Siddha system of medicine for infectious diseases affecting the hepatic system and renal system. In traditional medicine, root and leaf are used as diuretic, antipyretic, blood purification as well as to strengthen the stomach. The dry root of chicory is used as a substitute of coffee powder, and it is widely distributed in Asia and Europe (Bais *et al.*, 2001).

The best method for obtaining a large quantity of homogenous plants is by *in vitro* propagation (Pierik, 1987). For the supply of the broad range of plants having medicinal properties, the preliminary step is production of enormous numbers of plant material with low cost. Several reports have been proved the ability of regeneration of *C. intybus* from various parts of plants Margara and Rancillac (1966), leaf (Toponi 1963; Eung *et al.*, 1999), floral stem Bouriquet and Vasseur (1973), and petiole (Wagner and Eneva, 1996; Cadalen *et al.*, 2010), and protoplast of *C. intybus*. From the tissue culture technique, one can speed up the mass multiplication rate and a large number of the healthy and homogenous plantlets were produced by 10,000 times (Rao *et al.*, 1996).

Studies on *in vitro* propagation of *C. intybus* were performed on leaf explant (Rafsanjani *et al.*, 2011; Dolinski and Olek, 2013 and Rehaman *et al.*, 2001). The similar method was developed for the regeneration of plantlets containing 2 µm indole-3-acetic acid (IAA) + 5 µm kinetin (KN) and 1000 mg casein hydrolysate (CH) in witloof chicory Rehman *et al.* (2003). To produce a large number of uniform plants through mature rapid multiplication of *C. intybus*, the present work was carried out to develop a reproducible procedure for inducing indirect regeneration

of the plant from *in vitro* leaf explants and the anatomical studies of the various developmental stages of callogenesis.

MATERIALS AND METHODS

Plant Explants for the Culture

The *C. intybus* L. seeds were imported from Gujarat. Further, marketed by spring Haven Plot no: 3. Udyognagar; district Jamna Nagar India; and were grown in college garden, NMAMIT, Nitte, Karkala Taluk, South Kanara, Karnataka state, during the period of July 2011-september 2014. The germinated plantlets after 2½ months were used as the starting material for inoculation.

Sterilization of the Explants

The fresh and tender leaf was selected and surface sterilized by removing dust particles. Young tender leaves were positioned beneath running tap water for 45 min and washed thoroughly with a mixture of 2 drops of tween 20 (100 ml of distilled water) for 10 min followed by means of thorough washing with double distilled water to get rid of extra traces of germicidal agent. Leaves were immersed in 70% ethyle alcohol for 1 min. Further, rinsed with the double distilled water. Then, the explants are surface sterilized with commonly used 0.1% mercuric chloride ($HgCl_2$) for 8 min. After surface sterilization, these explants are washed with autoclaved distilled water. Disinfected leaves were trimmed properly and cut into small pieces including midrib region (1 cm) and aseptically inoculated to callus induction media (Figure 1a).

Nutrient Media and Culture Conditions

Murashige and Skoog (MS) media accomplished with 22 g/L sucrose (Himedia, India) was used as basal media. Solidified agar (0.8%) (Himedia, India) and the pH were maintained between 5.6-5.8 using 1 N sodium hydroxide or 1 N hydrochloric acid. The MS nutrient medium was autoclaved at 121°C for 15 min. Culture conditions viz.; 25°C ± 2°C and a normal photoperiod of 16/8 h (light/dark) are necessary for the growth. Daylight regimes were maintained at 3000 lux, and light is provided by a fluorescent lamp with 65-70% relative humidity.

In Vitro Culture for Callus Induction and *In Vitro* Regeneration

Tender leaf explants were inoculated to MS nutrient media with various concentrations of naphthalene acetic acid (NAA) (0.5-2.75 mg/l) and 6-benzyle adenine (BAP) (0.25 mg/l and 0.375 mg/l) for initiation and formation of the abundant callus. 10 replications were considered for every treatment. Individual explants were inoculated to the test tube, and 4-5 explants were inoculated into the culture bottles aseptically for the proliferation. After 15 days, grown callus was subcultured to the MS media including hormones 0.5-2.5 mg/l KN + 0.5 mg/l IAA + 500 mg of CH. MA-MI represents the concentrations of growth hormones. In this, KN concentrations were varied in each treatment from 0.5 to 2.5 mg/l, whereas other two growth factors such as IAA and CH in all the nine treatment from MA to MI kept constant throughout the experiment. Shoots without roots were detached from the regenerates and places onto the rhizogenesis medium, i.e., MS medium along with IBA 0.5 mg/l+ NAA + 0.5 mg/l. Rooted individual plantlets are separated out and carefully transplanted into pot under humid condition.

Histological Studies of Callogenesis

The callus at different stages of growth was used, i.e., 7th, 14th, 20th, and 45th day and direct organogenesis of the 15th day culture. The samples were fixed in standard fixative Carnoy's "B" fluid (Chloroform - 30 ml; absolute alcohol - 60 ml; and glacial acetic acid - 10 ml). Fixed materials were dehydrated serially using alcohol and butanol, which replaces the water in the material. A mixture of paraffin wax and bee's wax of melting point 58-60°C was used for infiltration and embedding. Uniformly thin sections of 10 µm thickness were cut using Leica RM 2145 rotary microtome. Toluidine blue stain was used to localize nucleic acids, and polysaccharide Schiff's reagent was used to localize polysaccharides, respectively.

RESULTS AND DISCUSSION

Callus Formation from the Leaf Explants

In the first stage, the leaf explants from the 2½ months old leaf explants, from the field condition was inoculated gentle on MS nutrient medium supplemented with a variety of combinations and concentrations of NAA and BAP. The concentration of NAA was varied from 0.5-2.75 mg/l and for BAP it was 0.25 mg/l and 0.375 mg/l. The leaf started enlarging after 4 days of inoculation. The callus initiation from cut ends of leaf explants occurred about 7 days after culture initiation (Figure 1b) in MS nutrient medium. The callus induction was observed at the cut ends of the leaf explants. It is also noticed that midrib portion showed better response toward callus induction compared to remaining cut ends of the leaf explants. The growth of the callus enlarged very rapidly and it covered the whole surface of the leaf explants (Figure 1c). The embryogenic callus consisted of a semi-transparent mass of greenish color cells (Mungole *et al*., 2009). The calli were maintained in the same media until 11 days.

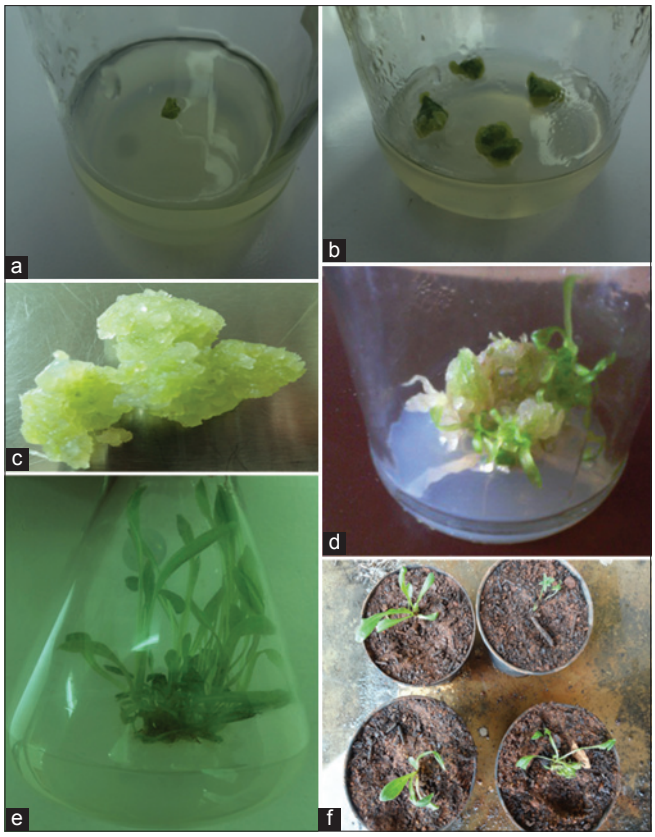

Figure 1: Shoot regeneration and plantlet establishments in pots from leaf explant. (a) Inoculation of leaf explant. (b) Callus initiation at the cut ends of the leaf explant after 7 days if incubation. (c) Callus multiplication after 14 days of incubation. (d) Shoot initiation from callus. (e) Multiple shoot formation. (f) Well-established plantlets in pots. Potting mixture consists of sand and soil (1:1)

The subculture was done on the 11th day of the culture initiation. The best callus formation and growth rate of the callus was observed in the MS nutrient medium supplemented with 1.5 mg/l NAA in combination with the 0.25 mg/l BAP (Table 1). The calli formed was loose friable and soft mass of the cells with greenish color. Similar calli were reported by Arif *et al.*, 2014 in Dianthus (*Dianthus caryophyllus* L.). The fresh weight of the callus was 27.65g. Callus formation and proliferation in the medium containing higher BAP concentration 0.375 mg/l lead to the poor development of the callus. However, Vermeulan *et al.*, 1993 induced microcallus from the leaf explant of *C. intybus* in the liquid media along with 1.0 mg/l NAA + 0.5 mg/l 2,4D + 1 mg/l BAP. Wagner and Gailing (1996) achieved the best callus production in the media in addition with 2.0 mg/l IAA + 0.26 mg/l of BAP.

Shoot Regeneration from the Callus

After transferring, the embryogenic callus to MS nutrient media containing (2 mg/l KN + 0.5 mg/l IAA + 500 mg/l CH), shoot primordia, and young shoots were initiated (Figure 1d). Regeneration of the primordia initiated after 16 days of culture incubation. The primordia changed into green, distinctive shoots in the presence of KN, IAA, and CH in the MS nutrient media. In all, the concentrations (KN 0.5-2.5 mg/l + 0.5 mg/l IAA + 500 mg/l CH, i.e., MA-MI) and combination shoot regeneration occurred rapidly (Figure 1e and f). Initially, 2-3 shoots were initiated in a small clump of the callus. A similar result was obtained by Velayutham *et al.*, (2006), and he achieved the regeneration process using leaf as explant and callus were initiated after 30 days of culture. The shoots were initiated in BAP rather than that of KN. The result obtained in BAP combination with that of IAA. Their response of shoots regeneration ranged from 30.2% to 89.1%. However, in our present work, the number of the young shoots per callus and height of the shoots (cm) were observed in all the concentration and combination after 30 days. The maximum height of the shoots were observed (9.66 cm) (Figure 2) per explants on MS medium with 2 mg/l KN + 0.5 mg/l IAA +500 mg/l of CH. In the present investigation, approximately 96.8% of the shoots were produced from the leaf explants. Multiple shoot induction was achieved in a combination of IAA 0.5 mg/l with KN 2 mg/l and 500 mg/l CH. However, Nandagopal and Kumari (2006) achieved suitable shoot formation in *C. intybus* by transferring the leaf-derived callus to (MS + B5) medium, with BAP, ADS, and KN as a growth regulator. After 45 Days of the culturing, it was observed that ADS played an important role in the percentage of the explants which produced callus and even number of shoots. About 93% of the shoots were obtained from the callus. Whereas in this study, the presence of KN and CH is necessary for the shoot formation from the leaf-derived calli, since the calli which were placed in MS medium with low concentration of KN and CH failed in regeneration into shoots Rehman *et al.* (2003). Moreover, we found that

Table 1: Weight gained (g) and morphological characters of calli obtained from leaf explants, subjected to various concentrations of NAA and BAP

NAA (mg/l)	BAP (mg/l)	Fresh weight of the callus (g/explants)	Color of the callus	Texture of the callus
0.5	0.25	4.2±0.473	Greenish white	Compact
0.75	0.25	4.3±0.571	Greenish white	Compact
1	0.25	10.52±1.246	Greenish	Loose friable
1.25	0.25	13.98±1.651	Greenish	Loose friable
1.5	0.25	27.65±0.850	Greenish	Loose friable
1.75	0.375	21.78±0.800	Greenish	Loose friable
2	0.375	17.55±1.145	Greenish	Loose friable
2.25	0.375	17.98±1.110	Greenish white	Loose friable
2.5	0.375	10.01±1.008	Greenish	Friable
2.75	0.375	4.9±1.013	Greenish white	Friable

NAA: Naphthalene acetic acid, BAP: 6-benzyle adenine

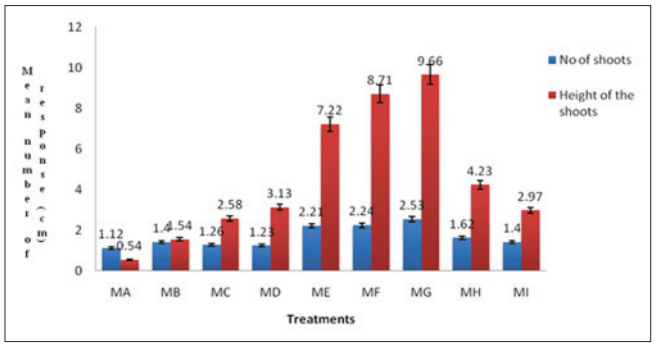

Figure 2: Graphical representation of number of shoots and height of shoots in which were obtained in various treatments MA to MI (0.5-2.5 mg/l kinetin + 0.5 mg/l acetic acid + 500 mg/l casein hydrolysate). Value represents error bar with percentage

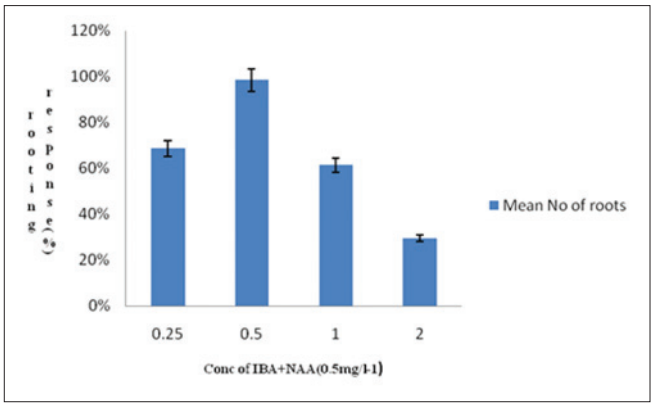

Figure 3: Effect of indole-3-acetic acid concentration (0.25, 0.5, 1, 2 mg/l) + naphthalene acetic acid 0.5 mg/l on rooting in indirect regenerated shoots from leaf explants of *Cichorium intybus*

increase in the KN concentration and CH concentration lead to the decrease in the growth of the shoots. Dolinski and Olek (2013) used wild *C. intybus* plants to study micropropagation. Their study was to access capacity of a normal population of wild *C. intybus* (var. *Silvestre* Bisch). They examined 16 combinations and concentration of IAA and 2 ip. The majority of shoots (97%) were obtained from the callus containing 0.5 mg/l IAA + 4 mg/l 2 ip).

Root Induction

Individual small shoots were detached aseptically and inoculated into rooting medium incorporated with different concentration of IBA (0.25-2.0 mg/l) and 0.5 mg/l NAA, which was kept constant. A linear increase in root induction was seen for 0.5 mg/l NAA + 0.5 mg/l concentration, The IBA and NAA growth regulators responded well in inducing the roots of the two hormones at the different concentration tested for the induction of the root. The maximum response showed for about 98% and acclimatized (Figure 3). Similar results were reported by Tiwari *et al*. (2000) and Yu *et al*. (2000).

Histological Studies of the *C. intybus* Callus Tissue

In our present investigation, the early stages of callus formation it was found that the parenchyma cells of the callus tissue protruded out to form the meristematic activity. A similar type of observation is seen in coconut by Verdeil *et al*., 1994, 2001. The protruded cells formed an undifferentiated mass of the cells or clumps of the cells which were developed on the surface of the leaf tissue after the 7th day of inoculation of the leaf explant (Figure 4a and b). Histological analysis of organogenesis from the callus culture of black pepper was reported by Sujatha *et al*. (2003). The division and growth of callus cells continued for some time resulting in the further multiplication of the callus (Figure 4c). The tracheids appeared in the 14th day callus cells along with the parenchyma cells (Figure 4d). The cells which appeared was darkly stained and tracheids which appeared scattered entirely in between the parenchymatous cells the derivatives started appearing and started a further division of the cells very quick forming globular type structure (Figure 4e and f). These globular structures showed high localization of polysaccharides. The callus developed on the MS medium containing NAA (1.5 mg/l), and BAP (0.25 mg/l) showed numerous meristematic regions. The succeeding development of vascular nodules in the mass of tissue indicated the formation of shoot meristems. Though the callus was very friable, the cells were tightly associated with each other, and it was not clearly differentiated. The origin of single shoot primordia was observed (Figure 4g and h). The callus with numerous vascular nodules, on culturing in the shooting medium developed. Histological analysis shows that the primordial shoots regenerated from the leaf-derived callus showed meristems in between. In direct regeneration single meristemoids and primordial shoots developed directly within 30 days after inoculation (Figure 4i and j). Kumar and Nandi (2015) studied histology of organogenic callus from internodes explants of *Asteracantha longifloia* Nees. The development of nodules in the callus indicated the presence of shoot meristems. The shoot primordia were induced from the callus which is derived from internodes.

CONCLUSIONS

The standardized protocols for plantlet regeneration of leaf explants of *C. intybus* were mentioned. Plantlets survived in pots were approximately 92%, and field is 73% a histological data of 7th, 14th, 20th, and 45th for

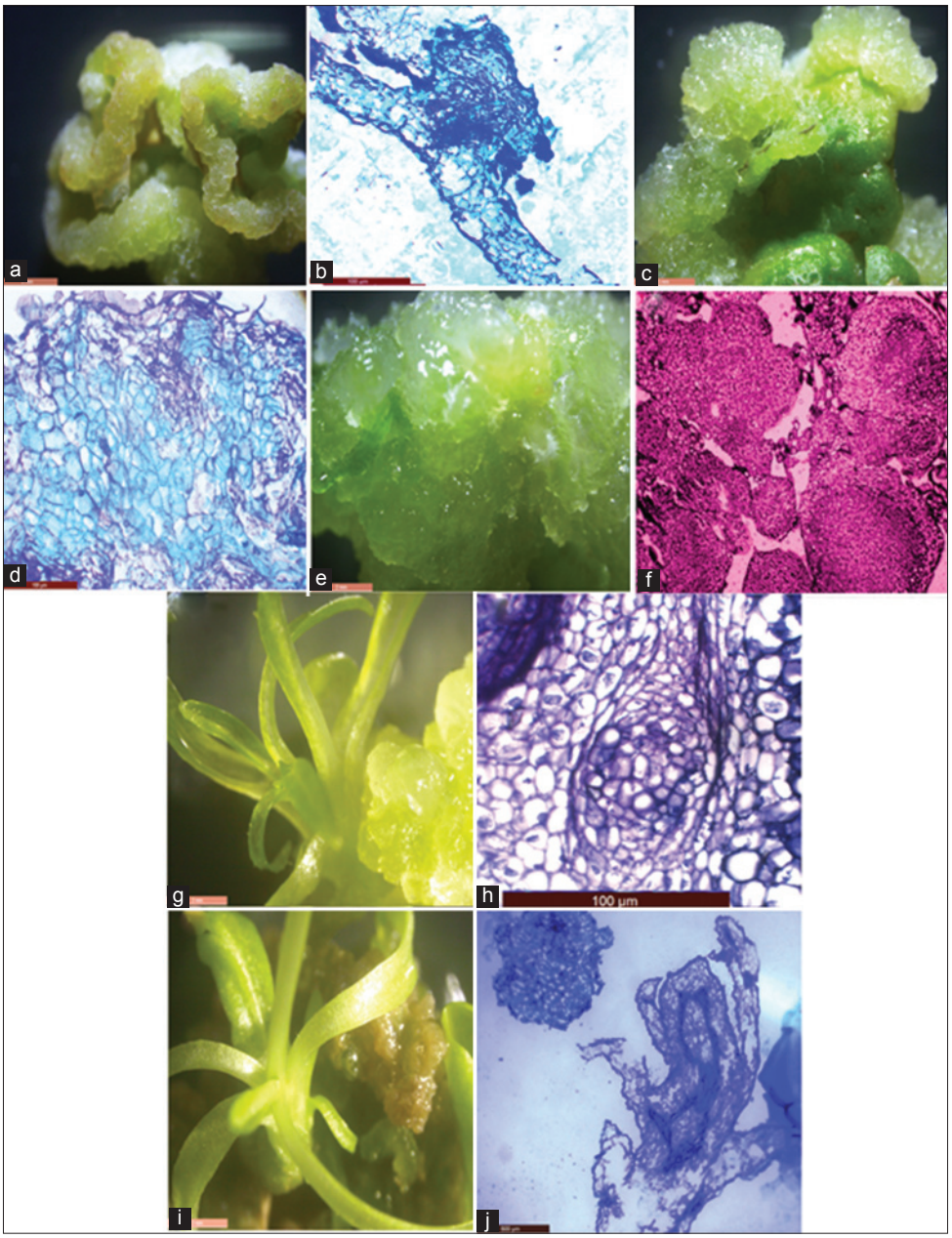

Figure 4: Histology of developmental stages of shoot regeneration. (a) 7 days old callus. (b) Transverse section (T.S) of 7 days old callus showing callus initiation from midrib of leaf explant. (c) Callus induction after 14 days. (d) T.S of callus showing the undifferentiated mass of cell and tracheids. (e) 20 days old callus. (f) T.S of calli showing meristematic zone formation (stained with polysaccharide Schiff's stain). (g) Shoot initiation after 45 days of callus incubation. (h) T.S showing initiation of the apical meristem. (i) Direct regeneration of shoots from leaf explants. (j) T.S showing apical meristem with leaf primordia

apical meristems formation from its organogenic callus is extremely reproducible and consistent.

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