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Adenine sulphate induced high frequency shoot organogenesis in callus and *in vitro* flowering of *Cichorium intybus* L. cv. Focus - a potent medicinal plant

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ABSTRACT

An efficient protocol has been developed for the *in vitro* plant regeneration and flowering achieved from young leaf explants of chicory (*Cichorium intybus* L. cv. Focus). The callus induction and shoot multiplication was obtained on Murashige and Skoog (MS) and Gamborgs (B_5) media supplemented with different concentrations and combinations of Indole-3-acetic acid, 6-Benzylaminopurine, 6-Furfurylaminopurine and Adenine sulphate. The highest percentage of callus induction and multiple shoots proliferation was observed on MS+B_5 medium containing 6-benzylaminopurine (6.66 µM) + indole-3-acetic acid (2.852 µM) + adenine sulphate (1.360 µM). For root induction regenerated shoots were transferred to MS+B_5 medium containing Indole-3-acetic acid, Indole-3-butyric acid and α-Naphthalene acetic acid that were developed roots. *In vitro* flowers were also noticed in the *in vitro* raised plantlets in the same medium under 16 h light and 8 h dark condition. The regenerated plantlets have been successfully established in vermiculite and subsequently shifted to the field.

Key words: leaf callus; growth regulators; plant regeneration; *in vitro* flowering; hardening.

IZVLEČEK

USPEŠNO IN VITRO INDUCIRANJE ORGANOGENEZE POGANJKOV RADIČA *Cichorium intybus* L. cv. Focus Z ADENIN SULFATOM

Razvita je bila metoda uspešne *in vitro* regeneracije radiča (*Cichorium intybus* L. cv. Focus) iz listnih delov; doseženo je bilo cvetenje regeneriranih rastlin. Induciranje kalusa in razmnoževanje poganjkov sta bili doseženi z Murashige in Skoog (MS) ter Gamborgovim (B_5) gojiščem, dopolnjenima z različnimi koncentracijami indol-3-ocetne kisline, 6-benzilaminopurina, 6-furfurilaminopurina in adenin sulfata. Največji odstotek induciranja kalusa ter največje število poganjkov sta bila dosežena na gojišču MS+B_5, ki je vsebovalo 6-benzilaminopurin (6.66 µM) + indol-3-ocetno kislino (2.852 µM) + adenin sulfat (1.360 µM). Za

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indukcijo korenin na poganjkih so ti bili prenešeni na gojišče MS+B_5, ki je vsebovalo indol-3-ocetno kislino, indol-3-masleno kislino in α-naftalen ocetno kislino, kar je omogočilo uspešno zasnovo korenin. Opazili smo tudi *in vitro* cvetove pri *in vitro* vzgojenih rastlinicah v istem gojišču, pri razmerah 16 h osvetlitve in 8 h teme. Rast regeneriranih rastlinic se je uspešno nadaljevala v vermikulitu in kasneje po presaditvi tudi na prostem.

Ključne besede: listni kalus; rastni regulatorji; regeneracija rastlin; *in vitro* cvetenje; utrditev.

1 INTRODUCTION

Chicory (*Cichorium intybus* L.) is a medicinally important plant that belongs to the family Asteraceae. Root, leaf and seed contains a number of medicinally important compounds such as inulin, sesquiterpene lactones, coumarins, flavonoids and vitamins. It is used as antihepatotoxic, antiulcerogenic, antiinflammatory, appetizer, digestive, stomachic, depurative, diuretic (Varotto *et al*., 1997). Recent pharmacological investigation of the root extract of this plant revealed immunomodulator and anticancer properties (Angelina *et al*., 1999).

Previous study reported by Varotto *et al*. (2000) on plant regeneration from leaf-protoplasts derived callus has been obtained in Italian red chicory (*C. intybus*) on medium containing 0.1 mg l^{-1} NAA. Rehman *et al*. (2003) developed a protocol for the regeneration of plantlets from leaf explants of witloof chicory. The obtained regeneration via callus on modified MS semisolid medium containing 2 µM IAA in combination with 5 µM KIN and 1000 mg l^{-1} casein hydrolyzate.

Flowering is considered as a complex process regulated by external and internal factors. In many plants, *in vitro* flowering were normally achieved by the application of exogenous hormones to the culture medium. The ability of explants to form flowers *in vitro* depends on numerous internal, chemical and physical factors and virtually all these interact in various complex and unpredicted ways (Tran than van, 1973; Scorza and Janick, 1980; Croes *et al*., 1985; Compton and Vielleux, 1992). Combination of genetic and environmental factors also plays a role in flowering response *in vitro* (Tisserat and Galletta, 1993). Flowering *in vitro* can occur on explants from flowering plants of some woody species (Scorza, 1982).

Cichorium intybus is a biennial plant whose requirements for floral initiation are not fully understood. Hartman (1956) and Harrington *et al*. (1959) showed that chicory plants require both vernalization and long days to flower. In order to induce florally, chicory must undergo an exposure to low temperature (3 weeks at 4°C) that can be applied to the germinating seeds or to the entire plant (Rappaport and Wittwer, 1956). Studies of *in vitro* cultured explants from chicory roots initially led to the conclusion that the explants also must be vernalized before they will initiated inflorescences (Nitsch and Nitsch, 1964; Paulet and Nitsch, 1964). In contrast with these findings, it was found that the initial root explants do not always need to be vernalized (Margara, 1965; Margara and Rancillac, 1966). Mikou and Badila (1992) produced inflorescences under short-day conditions of 9 hr if long days of 16 hr were applied initially. This indicates that the duration of light commits the meristem to the subsequent formation of flower primordia. Under natural conditions, chicory plants

are vernalized during the winter, and subsequently bolt and flowers in the second growing season from May until August (Demeulemeester and De Proft, 1999).

The purpose of the study was to develop an efficient *in vitro* regeneration system from leaf derived callus of *Cichorium intybus* L. cv. Focus, an economically important medicinal plant and to induce flowering from the *in vitro* regenerated plants. In the present work, we have established a reproducible method for high frequency callus induction, regeneration and flowering from leaf segments, followed by successful establishment of regenerated plants in soil.

2 MATERIALS AND METHODS

2.1 Plant material

The seeds of Chicory (*Cichorium intybus* L. cv. Focus) obtained from Wageningen Agricultural University, Netherlands were used as initial explants for plant source.

2.2 Seed sterilization and germination

Seeds were surface cleaned by washing in running tap water for 30 min. Then they were washed in an agitated solution of liquid detergent (1% v/v Teepol) for 5 min and distilled water for 2-3 times. After thorough washing, the seeds were taken into the Laminar Flow Chamber where they were disinfected with 70% (v/v) ethanol for 60-70 s followed by treatment with 0.1% (w/v) aqueous mercuric chloride ($HgCl_2$) solution for 3-5 min and then rinsed 3-5 times in sterile distilled water. Finally, the seeds were germinated under sterile conditions on moist cotton in test tubes (150x25 mm). The tubes were incubated in dark initially for 48 h and after transferred to light conditions of 3000 lux (Philips, India) light intensity provided by cool white fluorescent tubes.

2.3 Basal medium and incubation condition

The basal medium used in this investigation consisted of MS (Murashige and Skoog, 1962) and B_5 medium (Gamborg *et al*., 1968) supplemented with 30 g $l^{-1}$and 20 g l^{-1} sucrose (Himedia, India), respectively. 0.7% and 0.68% agar (Himedia, India) was used as the gelling agent on respective media. The pH of all media were adjusted between 5.7 - 5.8 using 0.1N NaOH or 0.1N HCl before solidification. The media were autoclaved at 121°C for 15 minutes for sterilization. All cultures were incubated in a controlled temperature of 25 ± 2°C. IAA, NAA, IBA, BAP, KIN and ADS were the different hormone sources taken for the present study.

2.4 *In vitro* culture conditions and shoots regeneration

The young leaf from 7 days old seedlings were inoculated on MS and B_5 media. Different concentration and combinations of hormones including 5.71-17.13 µM IAA, 0.492-14.76 µM IBA, 0.537-16.11 µM NAA, 0.444-13.32 µM BAP, 0.464-13.92 µM KIN and 0.272- 8.15 µM ADS were tested. The pH of all media was adjusted between 5.7-5.8 before it was autoclaved for 15 min at 121°C. The cultures were incubated at 25 ±2°C under 16/8 h (light/dark) photoperiod provided by cool white fluorescent tubes with 3000 lux intensity and relative humidity of 60–70%. The effects of different combinations of growth regulators on callus formation and regeneration were recorded at regular intervals (Table 1). Shoot cuttings excised from the regenerants were transferred to MS+B_5 medium supplemented with 0.571-14.27 µM IAA, 0.537-16.11 µM IBA and 0.492-14.76 µM NAA to induce rooting. Rooted plantlets were transferred to sterile vermiculite supplemented with half-strength MS salts and incubated under saturated humidity conditions. After 2 wk of hardening, plants were transferred to pots and grown at 25 ± 2°C initially and subsequently established in the field condition.

Table 1: Effect of different concentrations of growth regulators on callusing, regeneration, number, height of regenerated shoots and flowers of C*ichorium intybus* L. cv. Focus.

Sl No	BAP(µM)	IAA(µM)	ADS(µM)	Callus percentage (mean ± SE)	Regeneration percentage (mean ± SE)	Number of shoots (mean ± SE)	Height of shoots (mean ± SE)	Number of flower/per shoot apex (mean ± SE)
1	2.22	0.571	1.360	57.3±1.65^{d}	54.0±2.49^{d}	16.3±1.08^{d}	3.63±0.10^{c}	-
2	4.44	1.142	1.360	83.0±1.88^{b}	79.0±b1.69^{b}	23.6±1.96^{bc}	5.90±0.24^{d}	1.69±0.11^{b}
3	6.66	2.852	1.360	94.3±0.98^{a}	93.0±bc1.36^{a}	33.0±1.69^{a}	7.53±0.13^{a}	5.21±0.9^{a}
4	8.88	5.710	1.360	76.0±0.94^{bc}	74.0±1.88b^{c}	25.6±0.54^{b}	6.73±0.11^{bc}	0.75±0.2^{c}
5	2.22	0.571	-	52.6±1.90^{e}	51.0±2.84^{de}	11.3±1.36^{e}	9.56±0.05^{d}	-
6	4.44	1.142	-	40.3±1.90^{f}	38.0±1.63^{f}	09.0±0.47^{ef}	3.00±0.10^{de}	-
7	6.66	2.852	-	37.0±2.05^{fg}	34.3±1.08^{fg}	07.6±0.54^{f}	2.83±1.13^{e}	0.52±0.52^{cd}
8	8.88	5.710	-	32.3±1.18^{g}	28.6±0.72^{g}	06.3±0.27^{fg}	2.33±0.13^{ef}	-
	KINµM	IAAµM						
1	2.32	0.571	1.360	56.0±1.24c	37.3±2.17^{e}	06.6±0.72^{e}	2.66±0.13^{e}	-
2	4.64	1.142	1.360	82.0±1.24^{ab}	75.6±2.41^{b}	18.6±1.51^{bc}	5.23±0.11^{b}	-
3	6.96	2.852	1.360	90.6±0.54^{a}	88.0±1.24^{a}	29.0±0.47^{a}	6.66±0.13^{a}	-
4	9.28	5.710	1.360	74.6±1.96^{b}	69.3±0.27^{bc}	21.6±1.44^{b}	5.00±0.23^{bc}	-
5	2.32	0.571	-	52.0±2.05^{cd}	48.3±0.36^{d}	09.0±1.47^{d}	3.50±0.40^{d}	-
6	4.64	1.142	-	39.0±1.24^{e}	37.3±2.17^{e}	6.66±0.72^{de}	2.66±0.13^{e}	-
7	6.96	2.852	-	30.3±0.54^{ef}	26.0±0.47^{f}	6.03±0.27^{ef}	2.50±0.23^{ef}	-
8	9.28	5.710	-	21.6±0.54^{f}	20.0±1.24^{fg}	0.50±0.47^{g}	2.16±0.36^{f}	-

Each value represents the mean ± standard error (S.E.) of ten replicates per treatment in three repeated experiments. Means within a row followed by the same letters are not significant at P=0.05 according to DMRT.

2.5 Statistical analysis

The percentage of response, callus induction frequency, regeneration frequency of callus, number of shoots, shoot length, number of roots, root length and number of flowers were monitored as growth parameters. Data of three independent experiments represented by 10 replicates from each experiment were subjected to statistical analysis (Mean SE), according to New Duncan's Multiple Range Test (Gomez and Gomez, 1976).

3 RESULTS

3.1 Callus induction and shoot regeneration

Callus formation from leaf explants could be observed in all hormone combinations after 10-15 days. Young leaf from the 7 days old seedlings exhibited higher response

for callus induction when cultured on MS+B5 medium supplemented with different combinations and concentrations of BAP, IAA, KIN and with or with out ADS. The callus obtained with MS+B5 medium supplemented with 2.22-8.80 µM BAP, 0.571-5.710 µM IAA, 2.32-9.28 µM KIN and 1.360 µM ADS were greenish compact and pale yellow but were friable on MS+B5 medium supplemented with 2.32-9.28 µM KIN and 0.571-5.710 µM IAA. The percentage of callusing response was 57.3-94.3% on MS+B5 medium fortified with 2.22-8.88 µM BAP+0.571-5.710 µM IAA+1.360 µM ADS; it was 32.3 - 52.6% on 2.22-8.88 µM BAP+0.571-5.710 µM IAA. Without ADS in the medium, the callusing percentage was low. Maximum callusing (94.3%) was obtained on MS+B5 medium with 6.66 µM BAP, 2.852 µM IAA and 1.360 µM ADS (Table 1; Fig 1A).

Regeneration of shoot buds initiated in 30 days old callus. The buds transformed into green, healthy shoots in the presence of growth regulators in the medium on MS+B5 with 2.22 - 8.88 µM BAP + 0.571-5.710 µM IAA+1.360 µM ADS. The regeneration percentage was 54.0-93.0. The maximum regeneration of 93.6% was observed on MS+B5 medium with 6.66 µM BAP+2.852 µM IAA+1.360 µM ADS (Table 1). The number of shoots per callus and height of regenerants was monitored in all the combinations after 45 days. The number of shoots ranged between 16.3 and 33.0 on MS+B_5 media with 2.22-8.88 µM BAP+0.571-5.710 µM IAA+1.360 µM ADS and between 06.3-11.3 on MS+B5 media with 2.22-8.88 µM BAP+0.571-5.710 µM IAA. The maximum number of shoots 33.0 was observed on MS+B5 media fortified with 6.66 µM BAP+2.852 µM IAA+1.360 µM ADS. The shoot height ranged between 5.5 and 5.90 cms on media supplemented with ADS and between 2.66 and 3.50 cms on media without ADS. The maximum height of shoots was 9.5 cms on MS+B_5 media with 6.66 µM BAP+2.852 µM IAA+1.360 µM ADS on MS+B_5 with media 2.32-9.28 µM KIN+0.571-5.710 µM IAA+1.360 µM ADS the number or shoots ranged between 16.3 and 33.0 and the shoot height between 5.90 and 9.56 cms (Table 1; Fig 1 B, C) MS+B5 basal medium supplemented with 6.66 µM BAP+2.852 µM IAA+6.66 µM BAP+1.360 µM induced the best callus and shoot- forming response. These observations indicate that these media are at concentrations favorable for promoting callus and shoot proliferation in chicory cv. Focus.

3.2 Root induction and *in vitro* flowering

For rooting of 45 days old *in vitro* raised callus regenerated shoots, different concentrations of (02.85-14.27 µM) IAA, (2.460-1230 µM) IBA and (02.69-13.43 µM) NAA were tested. All the hormones responded well in inducing the roots (Fig. 1C, Table 2). Of the three hormones at different concentrations tested for root induction, the best response was obtained with 04.92 µM IBA, 05.71 µM IAA and 05.37 µM NAA. Maximum length of root was obtained with IBA (85.6 mm) followed by IAA (77.0 mm) and NAA (61.0 mm) (Table 2). The maximum number of flowers (5) was observed on MS+B_5 medium supplemented with 6.66 µM BAP, 2.852µM IAA and 1.360 µM ADS. These results indicate that without ADS in the culture medium, the number of flowers (1) produced was low. This result indicates that the media supplemented with ADS favours high frequency of *in vitro* flowering (5) in chicory plant (Table 1; Fig 1 E, F).

Table 2: Effect of different concentrations of auxins on rooting of microshoots and root length in C*ichorium intybus* L. cv. Focus.

S. No	Growth Regulators (µM)	Percentage of Response (Mean ± SE)	Root Length (mm) (Mean ± SE)
1	IAA 01.14	40.0 ± 0.94^{cd}	40.3 ± 0.72^{c}
	IAA 02.85	52.0 ± 0.47^{c}	41.6 ± 0.27^{bc}
	IAA 05.71	92.3 ± 0.27^{a}	77.0 ± 0.47^{a}
	IAA 08.56	79.9 ± 0.47^{ab}	44.0 ± 0.47^{b}
	IAA 11.42	69.6 ± 0.27^{a}	37.3 ± 0.27^{cd}
2	IBA 00.98	54.6 ± 1.18^{e}	54.0 ± 0.47^{d}
	IBA 02.46	66.3 ± 0.54^{d}	75.3 ± 0.27^{b}
	IBA 04.92	100 ± 0.00^{a}	85.6 ± 0.27^{a}
	IBA 07.38	92.0 ± 0.47^{ab}	69.6 ± 0.27^{bc}
	IBB 09.84	79.3 ± 0.72^{c}	50.6 ± 0.27^{de}
3	NAA 01.07	20.6 ± 0.27^{de}	23.3 ± 0.72^{d}
	NAA 02.69	52.1 ± 0.47^{d}	56.6 ± 0.27^{ab}
	NAA 05.37	78.6 ± 1.08^{a}	61.0 ± 0.00^{a}
	NAA 18.06	37.3 ± 1.18^{c}	42.0 ± 0.47^{c}
	NAA 10.74	21.3 ± 0.72^{d}	20.5 ± 0.27^{de}

Each value represents the mean ± standard error (S.E.) of ten replicates per treatment in three repeated experiments. Means within a row followed by the same letters are not significant at P=0.05 according to DMRT.

3.4 Establishment of plantlets

The plantlets with or without flower heads were successfully subjected to hardening on the foam cups for 15 days and then transferred to pots and grown at 25±2°C under 60- 70% relative humidity. 70-80% of plantlets were survived in the hardening procedure. These plants were then transferred to the field and were well established (Fig 1 D, G and H).

Figure 1: Regeneration and *in vitro* flowering of *Cichorium intybus* L. cv. Focus A - 15 days-old greenish compact callus from young leaf explant; B - multiple shoot formation from callus after 30 days; C - initiation of roots from isolated shoots after 10 days; D - well established plantlet hardening after 15 days; E - *in vitro* plantlet with flowers heads and rootlets; F - well established plantlet with flower heads; G - well established plantlet with flower heads hardening; H-Established plantlet with flower heads in the field.

4 DISCUSSION

The early studies reported that (Vermeulen *et* al., 993) micro calluses produced from the leaf tissue of *Cichorium intybus* in liquid medium supplemented with 1.0 mg/l NAA 0.5 mg/l 2, 4-D and 1mg/l BAP. Wagner and Gailing (1996) induced callus from the leaf vein segments of chicory on MS medium supplemented with 2.0 mg/l IAA and 0.26 mg/l BAP. Earlier reports on *Cichorium intybus* plant regeneration from the leaf explants were reported to be better for callus formation and shoot development (Eung *et al*., 1999). The leaf protoplasts derived callus has been obtained in Italian red chicory by Varotto *et al*. (2000). Rehman *et al*. (2001) cultured leaf segments of *Cichorium intybus* on MS medium supplemented with various growth regulators in different concentrations and obtained callus in the combination of 1.0 mg/l IAA with 5.0 mg/l KIN.

Similar results were reported in *Vitex* where they used 1.5 mg/l BAP in combination with 0.1 mg/l NAA (Thiruvengadam and Jayabalan, 2001). Various factors such as carbohydrates, growth regulators, light and pH of the culture medium are playing a major role in flowering (Heylon and Vendrig, 1998). Jumin and Ahmed (1999) recommended 0.01 mg/l BAP to induce flowers in *Murraya.* Stephen and Jayabalan (1998) induced maximum number of flowers from *Coriandrum* on culture with 0.15 mg/ l NAA and 0.5 mg/l GA_3. Patil *et al*. (1993) indicated that the exogenous cytokinin stimulates flowering by activation of endogenous cytokinin in ascending xylem sap. Earlier studies indicated that during budding and early flowering stages the basil oil was rich in monoterpenes (Lemberkovics *et al*., 1998). *In vitro* flowering in sunflower was also reported with low concentration of sucrose and 1 mg/l each of NAA and GA_3, and 0.5 mg/l activated charcoal (Patil *et al*., 1993). In our experiment high frequency *in vitro* flowering was achieved without GA_3 in the culture medium. However, increase in sucrose concentration (30 g/l) in the culture medium did not result in flowering in groundnut (Narasimhalu *et al*., 1984). But, in our investigation *in vitro* flowering was observed with higher sucrose concentration (30 g/l).

In chicory *in vitro* flowering was also influenced by the addition of 5-azacytidine, a DNA demethylation agent (Demeulemeester *et al*., 1999), putrescine and silver nitrate (Bais *et al*., 2000). In India, chicory cv. Focus flower initiation usually takes place during July-September and flowering takes almost after 521-551 days after planting. In the present investigation, flower production was observed from the cultured shoots after 45 days of culture. More number of flowers was observed in the hardened plants, which were growing in the field than that of normal plants. To our knowledge this is the first report on *in vitro* callus induction, plantlet regeneration and flowering of chicory cv. Focus.

Chicory flower contains a number of medicinally important compounds such as sesquiterpene lactones, coumarins, flavonoids, vitamins and also used as dye. Since chicory is a absolute long day plant, during the first year the plant shows only the vegetative growth and produces flowers and completes its life cycle in the second year (Varotto *et al*., 2000). In our experiment, *in vitro* flowering was initiated after 45 days of culture that reduces the breeding cycle and thus plays a key role in the field of agriculture, horticulture and pharmaceuticals.

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