EFFECTIVENESS OF AUXIN INDUCED IN VITRO ROOT CULTURE IN CHICORY

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ABSTRACT

An efficient protocol has been developed for the root culture of (Cichorium intybus L. cv. Focus), the leaf and hypocotyl explants from 25 days old in vitro raised seedlings were cultured on half-strength Murashige and Skoog (MS) medium supplemented with different concentrations and combinations of Indole-3-acetic acid (IAA), Indole-3-butyric acid (IBA), α-Napthalenacetic acid (NAA). 0.5 mg/l NAA and 0.1 mg/l IBA induced highest percentage of rooting from matured leaf explants, under total dark condition. After three weeks well established roots were separated. Fresh root tissue, in amount of 0.5 was subcultured in half-strength MS liquid medium supplemented with 0.2 mg/l NAA and 0.5 mg/l IBA, under continuous agitation at 110 rpm and total dark condition. The biomass of root culture was increased to 5.820 g after 6 weeks of culture. The root culture was maintained up to the 8 weeks.

KEY WORDS: In vitro root culture, matured leaf, hypocotyl, Cichorium intybus L.

INTRODUCTION

Chicory (Cichorium intybus L.), a member of Asteraceae is an erect glandular biennial herb with a tuberous taproot and rosette of 30-70 leaves. The stem grows upto 90 cms height; the lower leaves are larger, pinnately lobed and covered with hairs. The upper leaves on the elongated stem are very much smaller and their bases clasping the stem. During the first year the plant shows only the vegetative growth and produces flowers and completes its life cycle in the second year since it is an absolute long day plant [23].

Chicory was cultivated for the dried roots of this plant, which were used as a substitute or adulterant in coffee powder [12]. Initially chicory was listed in the Weed List in 1939 [10], but because of its value as forage for sheep and cows, farmers grow it as forage plant. In recent years this plant is cultivated for its medicinal, culinary and other uses. Chicory is a hardy perennial plant and grows in almost any soil. The seeds are sown in May or June in drills about 1 inch deep, about 12 inches apart in the rows. The fresh root is bitter, with a milky Juice. To obtain roots of a larger size, the soil must be rich, light and well manured. Under cultivation the root becomes large and fleshy with a thick rind and is employed extensively when roasted and ground for blending with coffee [24].

The tuberous root of chicory 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 [6, 15]. Inulin is used to replace fat or sugar and reduce the calories of food. Due to the non-digestibility it is suitable for consumption by diabetics [17] and is used in inulin clearance test to measure GFR [25]. Recent pharmacological investigation of the root extract of this plant revealed immunomodulator, antitumor and anticancer properties [9, 14].

The early studies on auxin induced lateral root formation in chicory were mainly concerned with the time course and pattern of lateral root initiation in excised roots of chicory grown in the presence of auxins [26, 27]. It is important to note that in approximately 60% of the medicinal plants used in the traditional systems of medicine (Ayurveda, Siddha, Unani), roots are the principle material for drug preparation [1]. It is estimated that more than 95% of the wild and more than 70% of the plant drugs involved destructive harvesting [25]. Development of biotechnological methods such as micropropagation, cell/root and hairy root culture is one of the major solutions to circumvent these problems. On this line, development of fast growing root culture system offers unique opportunities for providing root drugs in the laboratory, without resorting to field cultivation. Moreover, development of root culture is highly advantageous, as it is an alternative method for clonal propagation and germplasm conservation [3]. Root cultures can be used in many ways including studies of carbohydrate metabolism, mineral nutrient requirements, essentiality of vitamins and other growth regulators, differentiation of the root apex and gravitropism. The advantage of using root cultures in that they grow rapidly are relatively easy to prepare and maintain, show a low level of variability and can be easily cloned to produce a large supply of experimental tissue [19]. The natural regeneration as well as conventional propagation of this plant overcome the several factors like poor seed set, seed germination, seed viability and root initiation. Being a long day plant, it enters in reproductive cycle in the second season with long photoperiod [4, 5]. The purpose of the study was to develop an efficient in vitro development of root cultures from leaf and hypocotyl explants of chicory, an economically important medicinal plant. In the present work, we have established a reproducible method for high frequency root induction and root growth from leaf and hypocotyls explants, followed by successful establishment of hairy root like normal roots on MS solid and liquid medium without Agrobacterium rhizogenes in chicory plant. To our knowledge this is the first report on influence of exogenous hormones on fast-growing normal root culture of Cichorium intybus L. cv. Focus.

MATERIALS AND METHODS

Plant material

The seeds of (Cichorium intybus L. cv. Focus) obtained from Wageningen Agricultural University, Netherlands were used to obtain the leaf and hypocotyl explants which were used for the present study.

Seed sterilization and germination

Seed germination was carried out using the method of [16]. 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 in sterile distilled water. Finally, the seeds were germinated under sterile conditions on moist cotton in test tubes (150 x 25 mm). The tubes were incubated in dark initially for 48 h and after transferred to light conditions of 3000 lux (Philips India Ltd, Mumbai) intensity provided by cool white fluorescent tubes.

Basal medium and incubation condition

The basal medium used in this investigation consisted of MS nutrient salts [13] with 30 g $l^{-1}$and 20 g l^{-1} sucrose (Hi-media Pvt Ltd, India), respectively. 0.7% and 0.68% agar (Hi-media Pvt Ltd, India) was used as the gelling agent in respective media. The pH of all media was 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 min for sterilization. All cultures were incubated in a controlled temperature of 25±2 °C. Indole-3-acetic acid (IAA), α-Naphthalene acetic acid (NAA), Indole-3-butyric acid (IBA), were the different hormone sources taken for the present study.

In vitro culture conditions and root initiation

The matured leaf and hypocotyl explants from 25 days old seedlings were inoculated on full and half-strength MS medium supplemented with varied concentration of different auxins, indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), α-Naphthalene acetic acid (NAA), were tested. A total of 10 explant were cultured in each petri dishes. About 30 explants were cultured in each experiment. These treatments were repeated three times. The cultures were incubated at 25±2°C under 16 h photoperiod provided by cool white fluorescent tubes with 3000 lux intensity or under total darkness and relative humidity of 50-60%. The effect of varied concentrations of different auxins on root formation were recorded at regular intervals (Table 1).

Root induction on MS liquid medium

After six weeks well, established roots were separated from the explants aseptically and the nutrient medium sticking on the roots was removed. Thereafter, roots were cut into 0.5-1.0 cm long segments and fresh root tissue (0.5g/fw) was subcultured into 80 ml aliquots of half-strength MS liquid medium supplemented with IAA, IBA and NAA (0.1- 2.0 mg/l) in 250 ml Erlen meyer flask. The cultures were kept under continous agitation at 110 rpm on a rotary shaker (Neolab, India) and incubated under light (16 h photoperiod) or total dark condition. The root growth in different auxin regimes was assessed in terms of fresh weight after six weeks of culture. Regular subculture was done by inoculating growing root tip (0.5-1.0 cm) in the optimal medium (half-strength MS liquid medium with 0.2 mg/l NAA and 0.5 mg/l IBA at 2-weeks interval. The root biomass was determined up to a period of six weeks.

Statistical analysis

The percentage of response, root induction frequency, number of roots, root length, fresh and dry weight of roots from leaf and hypocotyl explants, were monitored as growth parameters. Data of three independent experiments represented by 10 replicates from each experiment were subjected to statistical analysis (Mean±SE) and New Duncan's Multiple Range Test [8].

Table 1 Effect of different auxins in half-strength MS solid medium on root induction of *in vitro*-derived leaf and hypocotyl explants of *Cichorium intybus* L. cv. Focus, under total darkness

Auxin (mg/l)	Percentage of response (%) (mean ±S.E)		Mean number of roots/culture (mean ±S.E)		Mean root length/culture (cm) (mean ±S.E)	
	Leaf	Hypocotyl	Leaf	Hypocotyl	Leaf	Hypocotyl
NAA + IAA						
0.5 + 0.2	64.0^{hi}	47.0^{g}	2.33 ± 0.13^{h}	1.50 ± 0.10^{fg}	5.00 ±0.23^{g}	4.60 ± 0.24^{gh}
0.5 + 0.5	70.0^{f}	66.0^{d}	2.83 ± 1.13^{fg}	2.30 ± 0.50^{e}	5.80 ± 0.37^{f}	4.90 ± 0.61^{g}
0.5 +1.0	82.0^{bc}	71.0^{c}	5.90 ± 0.24^{d}	3.50 ± 0.40^{c}	6.20 ± 0.32^{ef}	5.23 ± 0.11^{f}
0.5 + 1.5	76.0^{d}	66.0^{d}	3.63 ± 0.10^{f}	2.30 ± 0.50^{e}	5.50 ± 0.46^{fg}	5.20 ± 0.42^{f}
0.5 + 2.0	65.0^{h}	53.0^{ef}	2.60 ± 0.13^{g}	1.80 ± 0.20^{f}	5.00 ± 0.23^{g}	4.90 ± 0.61^{de}
NAA + IBA						
0.5 + 0.2	69.0^{fg}	56.0^{e}	5.23 ± 0.13^{de}	2.83 ± 1.13^{d}	6.60 ± 0.17^{e}	5.73 ± 0.72^{de}
0.5 + 0.5	74.0^{de}	63.0^{cd}	11.30 ± 1.36^{a}	5.23 ± 0.11^{a}	7.51 ± 0.54^{d}	6.01 ± 0.32^{d}
0.5 + 1.0	94.0^{a}	89.0^{a}	9.00 ± 0.47^{b}	4.50 ± 0.50^{d}	10.30 ± 1.08^{a}	8.60 ± 0.50^{a}
0.5 + 1.5	83.0^{b}	78.0^{b}	7.60 ± 0.54^{c}	3.50 ± 0.40^{c}	9.00 ± 0.47^{b}	8.20 ± 0.31^{ad}
0.5 + 2.0	76.0^{d}	71.0^{c}	6.60 ± 0.72^{cd}	2.83 ± 1.13^{d}	8.60 ± 0.57^{bc}	7.50 ± 0.54^{c}

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.

Table 2 Effect of different auxins in half-strength MS liquid medium on root induction of *in vitro*-derived leaf and hypocotyl explants of *Cichorium litybus* L. cv. Focus, under total darkness.

Auxin (mg/l)	Frequency of response (%) (mean ± S.E)		Mean fresh weight (g) (mean ± S.E)	
	Leaf	Hypocotyl	Leaf	Hypocotyl
NAA+IAA				
0.2+0.2	30.3^{g}	12.2^{f}	1.273 ± 0.75^{fg}	0.360 ± 0.032^{f}
0.2+0.5	39.5^{ef}	15.1^{ef}	1.420 ± 0.34^{f}	0.425 ± 0.005^{ef}
0.2+1.0	78.2^{b}	36.4^{b}	4.538 ± 0.43^{b}	3.106 ± 0.010^{b}
0.2+1.5	42.3^{e}	18.2^{d}	2.525 ± 0.09^{e}	0.868 ± 0.007^{e}
NAA+IBA				
0.2+0.2	48.2^{d}	16.0^{e}	3.000 ± 0.30^{d}	1.120 ± 0013^{d}
0.2+0.5	89.1^{a}	43.2^{a}	5.820 ± 0.024^{a}	4.106 ± 0.011^{a}
0.2+1.0	52.0^{cd}	20.3^{cd}	3.775 ± 0.012^{c}	2.065 ± 0.013^{c}
0.2+1.5	56.7^{c}	22.7^{c}	2.890 ± 0.009^{de}	0.952 ± 0.005^{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.

RESULTS AND DISCUSSION

A. Root induction on MS solid medium

1. In the present study well established normal roots were produced from leaf and hypocotyl explants could be observed on all the combinations after three weeks. Matured leaf and hypocotyl from 25 days old seedlings exhibited higher response for root induction when cultured on MS solid medium supplemented with different combinations and concentrations of IAA, IBA and NAA. The combination of 0.5 mg/l NAA and 1.0 mg/l IBA, was found to be best suited for growth promotion. After three weeks of culture, frequency of root induction, number of roots, root length were assessed.

B. Root formation from different explants

1. The root cultures were not established by the root and young leaf explants, because the survival rate was very low due to high contamination, slow growth and development of both the explants. Thus, the matured leaf and hypocotyl explants were alternatively used to establish the root culture of chicory plant. Explants cultured on full-strength MS medium always induced profuse callusing which subsequently turned brownish and hindered initiation of roots. Therefore, explants were cultured on half-strength MS medium (Table 1, 2). Root initiated with (1-2 weeks) in leaf explants followed by hypocotyl explants (2-3 weeks) in most of the treatments, under total darkness. Where as, under light condition (16 h photoperiod), rooting was delayed (4-5 weeks) in both explants. A minimum of 5-10 roots in leaf and 4-8 roots in hypocotyl explants were observed. Even though rooting was preceded by callusing at various degrees in few auxin treatments on either explants, efficiency of rooting was more with less degree of callusing in leaf explants (Figure 1 A, C) than hypocotyls (Figure 1 B, D). Complete dark culture was more effective for the production of roots than light illumination culture (Table 1, 2). This result was agreed to the Eleutherococcus and Decalepis root culture which produced more adventitious roots in dark condition than light condition [11, 20]. Dark incubation favoring root induction may be due to the slow metabolism of endogenously applied auxin in the dark than light as stated by [18].

C. Effect of exogenous auxins on root production

1. Of the various auxins supplemented, the response of the explants was higher in a combination of low concentration of NAA with high concentration of IAA or IBA (Table 1). Leaf explants (89%) incubated in total darkness in half-strength MS medium supplemented with an optimum combination of NAA (0.2 mg/l) and IBA (0.5 mg/l) showed rapid induction of roots and higher growth (5.820g fw) (Table 2). After a period of 6 weeks NAA and IAA combinations suppressed the lateral root formation compared to NAA and IBA combinations. However, in both combinations of auxins significant increase in the length of roots (15 - 20 cm) was noticed. The influence of individual auxins on root growth was negligible. Unlike IAA and IBA, the roots initiated in medium containing NAA alone at high concentrations were thick, short and occasionally callused. These results suggest that the effect

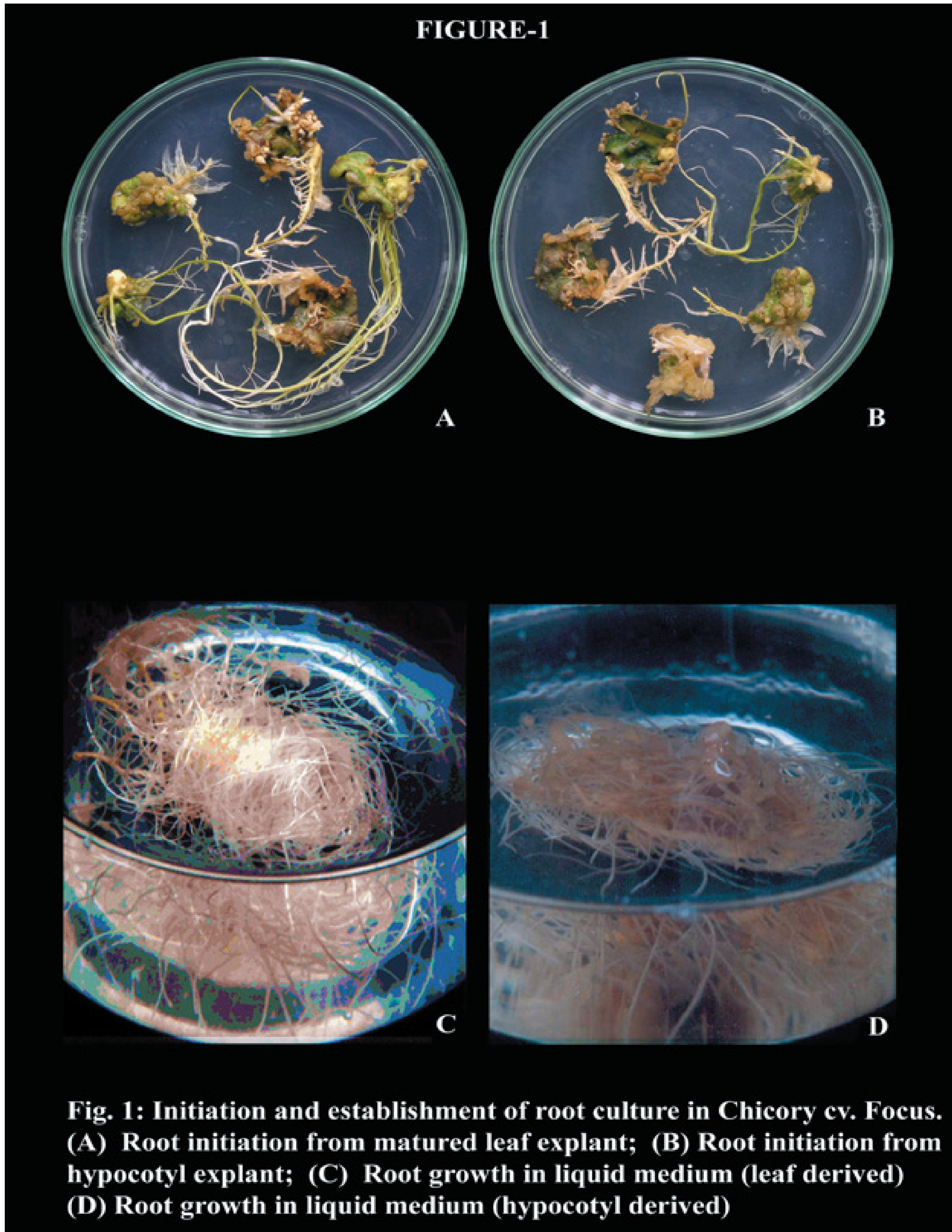

Fig. 1: Initiation and establishment of root culture in Chicory cv. Focus. (A) Root initiation from matured leaf explant; (B) Root initiation from hypocotyl explant; (C) Root growth in liquid medium (leaf derived) (D) Root growth in liquid medium (hypocotyl derived)

of auxin on adventitious root induction and elongation was depended on the plant types. In general, high level of auxin promoted the production of adventitious roots, although the auxin inhibited the elongation of root [2]. Contrary to our result, in Malus [7] IAA was effective for the production of roots and NAA strongly inhibited the growth of roots.

2. During serial subculture, reduced concentrations of auxin favored rapid and viable growth of roots (Table 2). NAA in subculture medium was found to be inevitable for profuse and rapid lateral root formation. The medium supplemented with 0.2 mg/l NAA and 0.5 mg/l IBA was best to produce maximum biomass (Table 2). Decrease in biomass was noticed in cultures due to low number of lateral roots because of the callusing of roots (Figure 1 B, D). It is important to note that in any system, the production of lateral roots is key factor for the rapid growth and is responsible for higher biomass.

3. Lateral root initiation on root segments in the present system was noticed on the root axis after 10th day of culture. The rapid growth started after 16 - 17 days by the elongation of lateral roots. Lateral roots elongated (2.0 - 3.5 cm) and formed a mat of roots at the end of 3 weeks (Figure 1 C, D). Maximum growth of roots was noticed at the 6th week of culture and later the growth was slowly declined. During the 8th week of culture, roots turned light brown, flaceid and brittle. For regular maintenance of root culture, it was found that root tip of 3-week-old culture were the best inoculum. The decline of root growth might be due to the accumulated endogenous auxin during each subculture. Most likely, the loss of root differentiation ability is one of the drawbacks of maintaining normal root culture for a long term as observed in chicory plant.

4. Hairy root culture systems are more efficient than normal root cultures, because of their genetic and biochemical stability over long periods and are ideal for introducing genes to elevate growth and secondary metabolism. However normal root culture system is an alternative method for those species which are recalcitrant to Agrobacterium rhizogenes infection. Before the establishment of first hairy root culture [22] normal root culture had been established for a number of species and demonstrated the ability to accumulate secondary products similar to those found in the roots of the parent plant [21]. But in most of the studies, callus-derived root culture systems were established. The conventional propagation is best with several factors like poor seed set, seed germination, seed viability and poor root initiation. Hence there is a need to apply the non-conventional propagation method for conservation and improvement of this species. Recently this plant has got important place in herbal medicine for the treatment of most common diseases like diabetes, inflammation, ulcer and cancer, etc. So, there is a need to man multiply the root system which has potential bioactive compounds which would be beneficial for the sustainable utilization of this medicinal plant for its bioactive ingredients, thereby which provides an alternative method rather than destroying whole plants.

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