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Full Length Research Paper

In vitro plant regeneration from embryogenic cell suspension culture of *Astragalus chrysochlorus* (Leguminoseae)

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***In vitro* plant regeneration was achieved from embryogenic cell suspension culture of *Astragalus chrysochlorus*. When 30-day-old aseptically grown seedlings were cultured on Murashige and Skoog (MS) medium containing 0.1 mg/l α-naphthaleneacetic acid (NAA) plus 1.0 mg/l 6-benzyladenine (BA), friable callus was formed within two weeks from the mesocotyl of the seedling. After three weeks, proliferated actively growing calli were transferred to MS liquid medium containing 2,4-dichlorophenoxyacetic acid (2,4-D), indole-3-acetic acid (IAA) or NAA and subcultured at two week intervals. After two weeks, induction of somatic embryos up to the torpedo stage occured at all tested concentrations of 2,4-D, IAA or NAA. Somatic embryos developed only in MS medium containing 0.5 mg/l IAA within two weeks and 2% of globular embryos were developed into the cotyledonary stage embryos. Eighty one percent of somatic embryos cultured in MS medium supplemented with 0.5 mg/l IAA were found to be diploid by flow cytometric analysis. Plantlet propagation was achieved on half strength MS liquid medium supplemented with 3% (w/v) sucrose after four weeks of culture. After a month on half strength MS medium [1.5% (w/v) sucrose and 0.8% (w/v) agar] 29 of 71 shoots developed into rooted plantlets.**

Key words: Somatic embryogenesis, propagation, indole-3-acetic acid, flow cytometry.

INTRODUCTION

Most of the *Astragalus* species are used in traditional medicine for their hepatoprotective, antioxidative, immunostimulative and antiviral properties (Ionkova et al., 1997). In Turkey, the genus *Astragalus* (Leguminoseae) is represented by approximately 439 species, 204 endemic, and various species are mainly used for gum production (Akan, 2000). Due to the economic and medicinal importance of the *Astragalus* species, they have been investigated for their chemical compounds (Çalış et al., 1997; Bedir et al., 1998, 1999; Yahara et al., 2000). *Astragalus chrysochlorus* Boiss. and Kotschy (2n = 16) is one of the endemic species in Turkey, growing in 32-36° meridian of northern Anatolia (Aytaç, 1997). In a previous study, cytotoxic activities of stem and root extracts of *A. chrysochlorus* were reported (Karagöz et al., 2007). Also, transformed root culture of *A. chrysochlorus* was established, and cytotoxic, antioxidant and phagocytic effects of the root extract of *A. chrysochlorus* were determined (Hasançebi, 2003).

Very few plant regeneration systems have been reported for *Astragalus* ssp. to date. Plants have been regenerated from hairy roots in *Astragalus sinicus* (Cho et al., 1998), from axillary buds in *Astragalus maximus* (Turgut-Kara and Ari, 2006) and from callus and protoplasts in *A. adsurgens* (Luo and Jia, 1998a, b). There are only few reports available on plant regeneration systems via somatic embryogenesis (Luo et al., 1999; Hou and Jia, 2004). Here, we report a protocol for plant regeneration from embryogenic cell suspension culture of endemic *A. chrysochlorus*. These results could provide a practical means of clonal propagation of this medicinally important plant and for further biotechnological applications.

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MATERIALS AND METHODS

Plant

Genetically pure seeds were collected and classified by Prof. Dr. Zeki Aytaç (Gazi University, Faculty of Arts and Science, Department of Biology, Turkey) and a voucher specimen was deposited in the herbarium at Istanbul University, Faculty of Science Herbarium (ISTF).

Germination of seeds and callus induction

Seeds were surface-sterilized in 70% alcohol for 1 min, then in 5% commercial bleach (Domestos) for 15 min, followed by three rinses for 15 min with sterile distilled water. Seeds were germinated aseptically in Petri dishes containing 25 ml of growth-regulator-free MS (Murashige and Skoog, 1962) medium supplemented with 3% (w/v) sucrose and solidified with 0.8% agar (w/v). The pH of MS medium was adjusted to 5.8 before sterilization by autoclaving at 121°C at 105 kPa for 20 min. The pH of MS medium was confirmed after sterilization. For callus induction, 30-day-old mesocotyl parts of seedlings were used. Seedlings were grown in culture tubes (Sigma, C-5916) containing 10 ml of MS medium supplemented with 0.1 mg/l α-naphthaleneacetic acid (NAA) and 1.0 mg/l 6-benzyladenine (BA). Callus proliferation was obtained on MS medium supplemented with 0.5 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) and subculture was performed at three week intervals. Twenty one day-old calli were used to establish cell suspension cultures after the first subculture. Seed germination, callus induction and subculture were carried out in a growth chamber illuminated with fluorescent light (*ca.* 1400 µmol m^{-2} s^{-1}) over a 16/8 day and night at 25 ± 2ºC.

Cell suspension culture

Twenty one day-old calli on MS medium supplemented with 0.5 mg/l 2,4-D were transferred to 50 ml Erlenmeyer flasks (~0.5 g fresh mass) containing 15 ml of MS liquid medium enriched with 0.5, 1.0, 3.0 mg/l concentrations of 2,4-D, indole-3-acetic acid (IAA) or NAA and agitated on a gyratory shaker (120 rpm, 25 ± 2°C, in dark). Cell suspensions were subcultured by adding 5 ml of the old suspension to 10 ml of fresh medium at two week intervals. Cell cultures were examined microscopically within 15th day for somatic embryo induction and determination of developmental stages. Embryogenic cell clumps were filtered through a 0.23 mm stainless steel sieve to determine somatic embryo number in 15-day-old suspension cultures.

After 15 day in suspension, filter-selected globular and larger embryos were transferred to MS liquid medium containing 0.5, 1.0, 3.0 mg/l concentrations of 2,4-D, IAA or NAA. Somatic embryos originated from MS liquid medium containing 0.5 mg/l IAA were used for cytokinin (0.1 mg/l BA, zeatin riboside (ZR) or kinetin) treatments and control group. 250 embryos were placed in 50 ml Erlenmeyer flasks containing 15 ml of liquid MS medium supplemented with growth regulators and cultures were agitated on a gyratory shaker (120 rpm, 25 ± 2°C, in the dark). In addition, the effect of different concentrations of sucrose (0.5-4.0%) for somatic embryo development were tested. After 15 day in suspensions, globular and larger somatic embryos which were originated from MS liquid medium containing 0.5 mg/l IAA were transferred to 25 ml half strength MS medium supplemented with sucrose (0.5-4.0%, w/v) and 0.8% (w/v) agar. Cultures were maintained in a growth chamber illuminated with fluorescent light and temperature conditions as mentioned above. Thirty embryos were placed in Petri dishes. Two replicates were used for each treatment, and the experiment was repeated two times.

Plantlet regeneration

After 15 day, the suspension culture was filtered and 15 cotyledonary embryos (5 mm in length) were selected for development and propagation and placed in 6-well culture plates containing 3 ml of half strength liquid MS medium supplemented with 3% (w/v) sucrose. Above mentioned growth chamber conditions were used for maintenance of cultures. After 30-day, propagated plantlets (10 mm length) without roots were excised and transfered to culture tubes (in vertical position) containing 10 ml of half strength MS medium with 1.5% (w/v) sucrose and solidified with 0.8% (w/v) agar for root development.

Histological investigations

For histological investigations, somatic embryos were fixed in acetic acid:ethanol (1:3, v/v) for 24 h, dehydrated in a graded series of alcohol and then embedded in parafin. Sections 4-5 µm thick were mounted on slides and stained with hematoxylin-eosin.

Ploidy of embryogenic cell suspension cultures

Ploidy level of embryos which were established from one year old embryo-genic callus were investigated by flow cytometric analysis. Globular and larger somatic embryos were collected from 9 different 25 day old suspension cultures (MS + 0.5 mg/l IAA). All selected embryos were chopped in a cold mortar containing a chopping buffer (45 mM magnesium chloride, 30 mM sodium citrate, 20 mM 4-morpholinepropane sulfonate and 1 mg/l Triton X-100, pH 7.0) (Galbraith et al., 1983). After 2 min, the cellular debris, consisting of finely minced tissue fragments, was passed through a nylon filter (pore size, 40 µm). The nuclei (~1300) in the filtrate were stained with propidium iodide. After 20 min of incubation in the dark, ploidy levels of somatic embryos were determined with flow cytometry (EPICS XL-MCL, Beckman Coulter).

Statistical analysis

To determine somatic embryo formation frequency, somatic embryos in 15 ml cultures were counted, and each experiment was repeated four times. Data were analyzed by one-way analysis of variance. The statistical significance of differences between means was estimated at the 5% level by the Tukey HSD test.

RESULTS AND DISCUSSION

Establishment of embryogenic callus

The mesocotyl parts of 30 day old seedlings produced friable and green callus on MS medium supplemented with 0.1 mg/l NAA and 1.0 mg/l BA after 15-day of culture (Figure 1a). Similarly, for embryogenic callus formation, the combination of NAA and BA has been used for *Astragalus adsurgens* Pall (Luo et al., 1999), some *Lilium* species (Tribulato et al., 1997) and *Hordeum vulgare* L. (Sahrawat and Chand, 2004). However, in *Astragalus melilotoides* the combination of NAA and BA was used for subsequent morphogenesis of somatic embryos which were derived from embryogenic calli (Hou and Jia, 2004). After induction of embryogenic callus, 2,4-D was used for proliferation purpose and *A. chrysochlorus* embryogenic

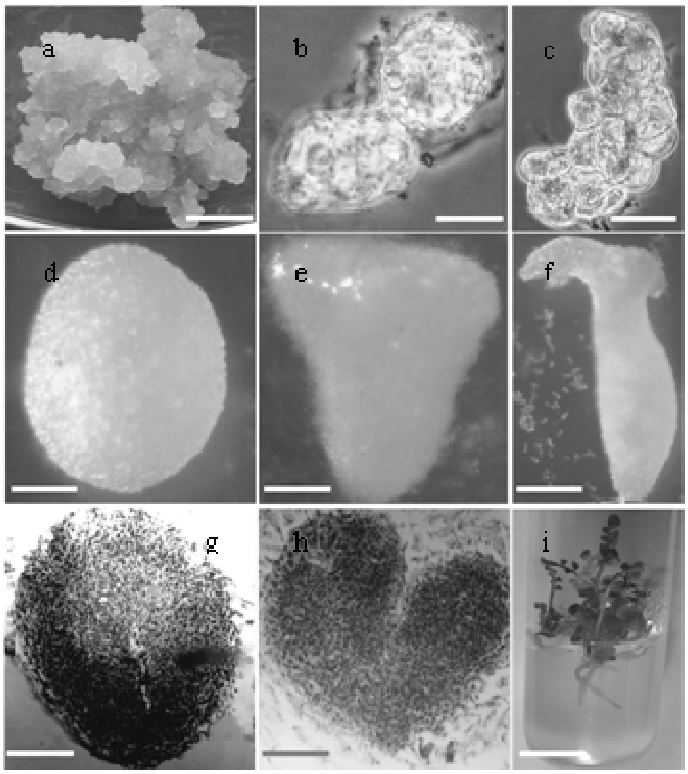

Figure 1. Somatic embryogenesis in *Astragalus chrysochlorus.* (a) embrygenic callus on 0.5 mg/l 2,4-D (bar: 6 mm), (b) spherical cells (bar: 40 µM), (c) proembryo (bar: 70 µM), (d) globular embryo (bar: 250 µM), (e) heart-shaped embryo (bar: 375 µM,), (f) cotyledonary embryo (bar: 500 µM), (g) longitudinal section of globular embryo (bar: 200 µM), (h) longitudinal section of heart-shaped embryo (bar: 300 µM), (i) rooted plant (bar: 15 mm).

callus had retained its morphogenetic potential for over one year by subculture at three week intervals on MS medium supplemented with 0.5 mg/l 2,4-D. Similarly, in carrot cell culture and *Triticum* durum callus culture, the use of 2,4-D maintained the long-term embryogenic capacity (Nomura and Komamine, 1985; Borrelli, 1991). In our study, 21 day old opaque, green and friable calli were used to establish the cell suspension cultures.

Effects of auxins on induction and number of somatic embryos

Induction of somatic embryogenesis from cell suspension cultures was achieved in all tested concentrations of auxins. Although, 3.0 mg/l IAA was the most effective (75.50 ± 0.64 somatic embryo/ml) (Table 1). In legumes, with a few exceptions, somatic embryogenesis has been induced in the presence of different types of auxins (Baker et al., 1994; Ahmed et al., 1996). Response of legumes to auxins varies significantly depending on the species and the type of the auxin used (Lakshmanan and Taji, 2000). For *A. adsurgens*, the high frequency of somatic embryo induction has been reported on MS medium supplemented with 0.1 mg/l NAA and 1.0 or 2.0 mg/l BA (Luo et al., 1999).

Table 1. Frequency of somatic embryo formation in MS-liquid medium supplemented with different 2,4-D, IAA and NAA concentrations in *Astragalus chrysochlorus.*

Growth regulators (mg/L)	Mean number of somatic embryos/ml*
0.0	0.0 ± 0.0 g
2,4-D (0.5)	24.00 ± 0.57e
2,4-D (1.0)	64.25 ± 0.75b
2,4-D (3.0)	19.75 ± 0.47e
IAA (0.5)	40.50 ± 1.32c
IAA (1.0)	66.75 ± 0.85b
IAA (3.0)	75.50 ± 0.64a
NAA (0.5)	13.25 ± 0.75f
NAA (1.0)	28.75 ± 1.31d
NAA (3.0)	41.75 ± 1.25c

*Values with the same letters are not significantly different (P<0.05). Results are expressed as means ± S.E.

Microscopic observations showed cell division on the second day of the suspension cultures. Two morphologically different types of cells were observed. The first was isodiametric (Figure 1b), the second elongated. Isodiametric cells divided further and resulted in the formation of cell clumps considered to be proembryos (Figure 1c). The proembryo divided further in multiple planes, which resulted in the formation of a globular embryo (Figure 1d, g). Globular embryos differentiated into heart (Figure 1e, h) and torpedo-shaped embryos, which later developed into cotyledonary stage embryos (Figure 1f) within first two weeks. Microscopic and histological observations confirmed the ontogeny of somatic embryo development in *A. chrysochlorus.* Somatic embryogenesis of *A. chrysochlorus* takes a relatively short time, since the total time required to obtain the first cotyledonary stage embryo is two weeks in cell suspension culture. However, in callus cultures of *A. adsurgens*, globular embryos developed further into heart- and torpedo-shaped embryos in two weeks, and embryos at the cotyledonary stage were observed after four weeks (Luo et al., 1999).

Effects of different media components on the development of somatic embryos

Many factors including the explant source, growth regulators (especially auxins and cytokinins) and other inductive factors (such as bioactive compounds, type of carbon source etc.) are responsible for effective somatic embryogenesis (Lakshmanan and Taji, 2000). In this study we have examined the effect of plant growth regulators (auxins and cytokinins) and sucrose on the development of somatic embryos in liquid MS and half

Table 2. Effects of growth regulators and sucrose on somatic embryo development in *Astragalus chrysochlorus* (Data taken after two weeks of culture when individual embryos could be separated from the cell suspension cultures).

Medium + growth regulator (mg/L)	Morphological observation
MS-L + 0.0	Root growth
MS-L + 2,4-D (0.5)	-
MS-L + 2,4-D (1.0)	-
MS-L + 2,4-D (3.0)	-
MS-L + IAA (0.5)	2% somatic embryos developed into cotyledonary stage embryos
MS-L + IAA (1.0)	-
MS-L + IAA (3.0)	-
MS-L + NAA (0.5)	Root growth
MS-L + NAA (1.0)	Root growth
MS-L + NAA (3.0)	Root growth
MS-L + BA (0.1)	Callusing
MS-L + ZR (0.1)	Callusing
MS-L + Kinetin (0.1)	Callusing
Medium + sucrose (%)	**Morphological observation**
½ MS-S + 0.0	-
½ MS-S + 0.5	Root growth, callusing
½ MS-S + 1.0	Root growth, callusing
½ MS-S + 1.5	Root growth, callusing
½ MS-S + 2.0	Root growth, callusing
½ MS-S + 2.5	-
½ MS-S + 3.0	-
½ MS-S + 4.0	-

MS-L: MS-Liquid medium including 3% sucrose.
½ MS-S: Half-strength MS solid medium without growth regulators.

strength solid MS medium, respectively, and we also used plant growth regulator free MS medium (Table 2). While tested concentrations of NAA induced only root regeneration from somatic embryos, cytokinins caused callusing (Table 2). Somatic embryos did not develop to cotyledonary stage on MS medium supplemented with all tested concentrations of 2,4-D, NAA and, 1.0 and 3.0 mg/l concentrations of IAA. Cotyledonary developmental stage (Figure 1f) was observed only at 0.5 mg/l IAA with the frequency of 2% (Table 2). Ribnicky et al. (2002) have reported that IAA was a critical factor for regulation of carrot embryogenesis and other instances of plant totipotency. In the present study, 2% of the torpedo-shaped embryos developed into cotyledonary-stage embryos on MS medium supplemented with 0.5 mg/l IAA. In most of the culture conditions, only root development was observed during the second week of cultures. The low frequency of cotyledonary stage embryos might be a result of this precocious root development, which could inhibit the development of the shoot pole. Inhibition of shoot pole development by early root formation is a problem encountered in several other plants (Tournand et al., 1983, Tejavathi et al., 2000). In contrast to our results, in many plant species, cytokinins have been used to support the growth of somatic embryos (Ammirato, 1983). In *A. chrysochlorus*, cytokinin treatment suppressed both maturation and germination of somatic embryos, and stimulated callusing.

The types and concentration of carbon source are important factors for induction of somatic embryogenesis (Lakshmanan and Taji, 2000; Tejavathi et al., 2000). Higher concentrations of sucrose are known to favour growth and development of the embryo (Tejavathi et al., 2000). In our study, the concentrations of sucrose tested caused callusing and root formation instead of somatic embryo development. Similar results have also been observed in soybean and flax (Tejavathi et al., 2000; Hofmann et al., 2004). However, sucrose has been found to be effective for somatic embryo development in peanut (Eapen and George, 1993). These findings suggest that the effects of sucrose on somatic embryo development might be species-dependent.

Planlet propagation

Plantlets were micropropagated from 15 day old cotyledonary embryos on half strength MS liquid medium supplemented with 3% (w/v) sucrose without growth regulators (71 axillary shoots were obtained from 15 embryo after four weeks). Axillary shoots derived from cotyledonary embryos were excised and transfered to solid half strength MS medium containing 1.5% (w/v)

sucrose, shoots developed and rooted. Twenty nine of 71 shoots (40.8%) rooted after 1 month (Figure 1i). Our results indicated that, although the formation of cotyledonary somatic embryos was low, micropropagation capacity of the shoots derived from somatic embryos was high. This shoot proliferation procedure through axillary buds derived from somatic embryos could provide an important way to propagate plants which have low regeneration capacity, like *Cajanus cajan* (Anbazhagan and Ganapathi, 1999) and flax (Tejavathi, 2000).

Ploidy of somatic embryos

The ploidy of the somatic embryos was investigated with flow cytometric analysis. Eighty one percent of globular and larger embryos derived from 25 day old cell suspension culture showed 2C DNA content (diploid) and 19% aneuploid DNA content. Similarly, in some plant species, there was no evidence of significant gain or loss of DNA in the embryogenic cell suspension cultures (Karlsson and Vasil, 1986; Binarova and Dolezel, 1988).

Conclusion

In the study presented, somatic embryos produced from cell cultures, as well as plant micropropagation of *A. chrysochlorus* cotyledonary embryos, has been achieved. Somatic embryogenesis via cell suspensions is an efficient regeneration system because cells or individual embryos can easily be handled and manipulated. This system might be suitable for further biotechnological applications, such as genetic transformation, clonal propagation of endemics, medicinal and commercially important plant species and production of artificial seeds.

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