



***In vitro* micro propagation, callus induction and shoot regeneration in safflower L. cv. Lesaf**

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Abstract

The present study was conducted to induce callus from different *in vitro* grown seedling explants of shoot, root and leaf segments in order to develop shoot buds and rooting from callus. The sterilized seeds were cultured on Murashige and Skoog medium, and then the explants were cultured from seedling and transferred to an MS medium supplemented with different concentrations of BAP, kinetin, NAA, and 2, 4-D growth regulator hormones. Among all explants, the leaf explants should produce significant callus induction (97.79 %) on MS + 1.0 mg l⁻¹ BAP medium. After 4 weeks, the obtained calluses were transferred to a new regeneration media with different concentrations of BAP and NAA and then the calluses were sub cultured on the same medium every 2 weeks. Overall, the maximum regeneration frequency was obtained from MS medium containing 0.1 mg l⁻¹ NAA and 1.0 mg l⁻¹ BAP.

Keywords: safflower; callus induction; shoot regeneration; 2, 4-D, BAP; Kinetin; NAA

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Introduction

Safflower (*Carthamus tinctorius* L.) is a member of the Compositae or Asteraceae family, which is an important oilseed crop grown in semi-arid regions and cultivated mainly for its seed

(Dilek et al., 2008). *Carthamus* L. genus consists of about 25 main species in the world. Species of *C. tinctorius* (safflower) corolla is known as an important crude drug in traditional Chinese medicine for promoting blood circulation and removing blood stasis. It has been used clinically in the prevention and treatment of cardio-vascular and thrombotic diseases (Bie, 2003; Jiang et al., 2005).

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Successful application of plant tissue culture techniques for crop improvement requires suitable plant regeneration methods. The success of *in vitro* culture depends mainly on the growth conditions of the source material (Ghasempour et al., 2012), medium composition, and culture conditions (Soheilikhah et al., 2013). Also, as an important tool, callus cultures are extremely important in plant biotechnology (Ghasempour et al., 2012). Callus induction and *in vitro* plantlet regeneration systems are optimized using cotyledonary explants by direct organogenesis (Neetika et al., 2005; Ghasempour, et al., 2007; Sharifi, et al., 2012) and direct somatic embryogenesis (Mandal and Gupta, 2003). Also, it is very important to increase the rate of multiplication frequency for genetic transformation studies of safflower. Furthermore, the interaction of explants and growth regulators was found to be significant in the earlier investigations on safflower tissue culture (Soheilikhah et al., 2013). The differences among different parts of the same plant may be attributed to the various levels of endogenous plant growth regulators of explants from different positions (Uranbey et al., 2005).

The purpose of the present study was to determine the most suitable concentrations and combination of growth regulators for improvement in callus induction and *in vitro* shoot and root regeneration efficiency in *Carthamus tinctorius* cv. Lesaf from cotyledon, hypocotyls and root segment explants of *in vitro* raised seedling.

Materials and Methods

Plant material

Certified seeds of cultivar of safflower (*Carthamus tinctorius* cv. Lesaf) were obtained from the experimental farms of the Agriculture Faculty of Razi University, Kermanshah, Iran, in September 2010. Lesaf cv. was the genotype used in the present study. This research was carried out in the Biotechnology and Plant Physiology Laboratory of the Biology Department, Faculty of Sciences, Razi University, Kermanshah, Iran during the period 2010 to 2011.

Surface sterilization

Seeds were surface sterilized with 0.1% mercuric chloride for 7 min followed by three 5 min washes in sterile distilled water. Then, seeds were germinated and grown on MS media implemented with 2% sucrose and 7% agar. The pH of medium was adjusted to 5.7 with 0.1 N NaOH or 1 N HCl then agar added at 7 g⁻¹ (w/v) before autoclaving for 20 min at 121 °C and 108 KPa). The cultures were kept in controlled environment at 23±2 °C and 16-8 hr light/dark period under fluorescent light (19.75 micromole m⁻² s⁻¹) and 80±10 relative humidity (Convicon G30 germinator).

Callus induction and subculture

A variety of explants, inducing cotyledons, hypocotyls, and root were excised from 10 to 15-day-old seedling and divided into 0.5-1.0 cm segments. These explants were cultured on MS-based medium supplemented with different concentrations and combinations of 2, 4-dichlorophenoxyacetic acid (2, 4-D), kinetin, BAP, and α-naphthaleneacetic acid (NAA) for callus formation. The obtained calluses were sub cultured to the same medium every 15 days. After two times of sub culturing, the growing states of calluses were compared.

Shoot regeneration

After two subcultures (for 4 weeks), calluses obtained from various explants were transferred to Petri dishes containing shoot regeneration MS medium supplemented with various concentrations and combinations of BAP and NAA with 2% sucrose and 0, 1, 3, and 5 mg l⁻¹ BAP and 0.1 mg l⁻¹ NAA. Cultures were incubated in growth cabinet at 25±1 °C under 16 hrs of photoperiod.

Experimental design

The implemented treatments had three replicates; each replicated petri dish contained 10 explants in order to induce callus. Also, the degree of significance was determined by analysis of variance (ANOVA) and the differences between the means were compared by Duncan's multiple

range tests using MSTAT-C computer programmed. Furthermore, before statistical analysis, data given in percentages were subjected to arcsine (\sqrt{X}) transformation (Snedecor and Cochran, 1967). Also, each described experiment was repeated at least twice and all results were pooled.

Table1
ANOVA based on factorial experiment for callus induction

Source of Variation	DF	Mean square
Explant (A)	2	2960.773**
Hormone (B)	7	16077.412**
(Explant*Hormone) AB	14	299.542**
Error	48	23.482
CV%		7.04

** Significant at $P \leq 0.01$

Frequencies of the callus induction and regeneration were determined as the percentage of explants and the percentage of calluses, respectively, ended in production of full shoot and regenerated plants.

$CIF = \frac{\text{No. of explants producing callus}}{\text{No. of explants plated}} \times 100$

$PRF (\%) = \frac{\text{No. of calluses regenerated plantlets}}{\text{No. of calluses plated for regeneration}} \times 100$

Results

Leaf, hypocotyls and roots explants which were cultured on MS medium with different concentrations of BAP, NAA, 2, 4-D, and KIN, as well as desolated combination of mentioned growth regulators were successfully propagated (Table 2). The best result in terms of percentage response (97.79%) and nature of the callus induction were obtained with the combination of BAP (1 mg l^{-1}) in case of leaf-explants after 25-30 days (Fig. 1 a). Also, about 96.97% of the hypocotyls of explants successfully produced callus with the combination of 2, 4-D (0.5 mg l^{-1}), NAA (0.5 mg l^{-1}) and BAP (0.5 mg l^{-1}) as above after 25-30 days (Fig. 1 b). The percentage response in case of root explants was observed quit low in comparison to the leaf and shoot tip explants. The best result in root explants,

callusing (82.00%) was observed (Table 2) with the combination of 2, 4-D (0.5 mg l^{-1}), NAA (0.5 mg l^{-1}) and BAP (0.5 mg l^{-1}) after 20-25 days (Fig. 1 c). On the basis of calluses obtained from all the three explants (leaf, hypocotyl and root), further studies were carried out for shoot regeneration capacity of the callus (Table 1).



Fig. 1. Callus induction in *Carthamus tinctorius* (Safflower); a, callus induction from leaf segment on MS medium containing 0.5 mg l^{-1} 2,4-D + 0.5 mg l^{-1} BAP + 0.5 mg l^{-1} NAA; b, callus induction from shoot segment on MS medium containing 0.5 mg l^{-1} 2,4-D + 0.5 mg l^{-1} BAP + 0.5 mg l^{-1} NAA; c, callus induction from root segment on MS medium containing 0.5 mg l^{-1} 2,4-D + 0.5 mg l^{-1} BAP + 0.5 mg l^{-1} NAA.

Table 2

Callus induction from Shoot, Root, and Leaf segments of *Carthamus tinctorius* grown on MS medium supplemented with various concentrations of 2,4-D, BAP, NAA and KIN after 25-30 d of culture

Plant growth regulators mg l ⁻¹	Leaf segment	Shoot segment	Root segment
2, 4-D (1)	54.53 ^{fg} ± 0.61	49.07 ^g ± 0.68	25.00 ^f ± 1.34
2, 4- D (1.5)	69.09 ^d ± 0.48	54.77 ^{fg} ± 0.61	37.50 ^h ± 0.89
BAP (1)	97.79 ^a ± 0.34	66.64 ^{def} ± 0.50	68.09 ^{de} ± 0.49
NAA (1)	85.79 ^{bc} ± 0.39	59.11 ^{defg} ± 0.56	56.57 ^{efg} ± 0.59
KIN (1)	93.54 ^{abc} ± 0.36	51.00 ^g ± 0.66	57.04 ^{defg} ± 0.59
Kin (0.1) + 2, 4- D (5)	65.97 ^{def} ± 0.51	65.27 ^{def} ± 0.51	63.71 ^{def} ± 0.53
BAP (3) + NAA (0.1)	83.96 ^c ± 0.39	89.60 ^{abc} ± 0.37	81.95 ^c ± 0.41
BAP (0.5) + NAA (0.5) + 2,4- D (0.5)	96.16 ^{ab} ± 0.35	96.97 ^{ab} ± 0.35	82.00 ^c ± 0.41

Data shown are mean ± SE of three experiments; each experiment consisted of 25 replicates. ** Significant at P ≤ 0.01

Shoots initiated from the calluses obtained from all the three explants were cultured on MS medium with different concentrations of BAP and NAA, alone or in combination. The best shooting (25.11%) was observed with MS medium supplemented with BAP (1 mg l⁻¹) and NAA (0.1 mg l⁻¹) after 8-10 weeks (Fig. II), which was therefore used for further regeneration and transformation experiments (Tables 3 and 4).

Discussion

Callus was induced in all media tested and significant differences were observed in the induction frequency between different plant growth regulators (Table 2). The Cytokinin concentrations significantly affected the percentage of shoot regeneration, shoot numbers, and shoot length (Bohidar et al., 2008). In contrast to the above results, our data showed that only lower concentrations of Cytokinin were significantly effective on the percentage of shoot regeneration (Table 4). Cytokinin concentration has been reported to be decisive for shoot proliferation and elongation of many medicinal plant species (Ghasempour et al., 2007; Ghasempour et al., 2012; Soheilikhah et al., 2013).

Various factors do affect induction, maintenance, and regeneration of somatic embryos (Ghasempour et al., 2012; Sharifi, et al., 2012), but these studies have followed the classical two step method: first, production of callus by using high concentrations of different hormones; therefore, reports for optimum callus induction were different. In the second step,

Table 3

ANOVA based on CRD experiment for Shoot regeneration

Source of Variation	Df	Mean square
Calluses	3	1075.706**
Error	8	36.642
CV%		19.92

*Significant at P ≤ 0.01

Table 4

Shoot regeneration from Callus of *Carthamus tinctorius* on MS medium supplemented with various amounts of BAP after 8-10 week of culture

Plant Growth Regulators (mg l ⁻¹)	Cultures Producing Shoots (Mean ± SE) (%)
BAP	
0	00.00 ^c ± 00.00
1	25.11 ^a ± 0.24
3	12.83 ^b ± 0.47
5	5.043 ^c ± 1.19

Values within a column followed by different letters are significantly different at P ≤ 0.01.

Cytokinins is included in the media containing very low or no auxin and in order to obtain shoot regeneration. The presence of even minimum callus interphase poses the problem of genetic instability (Gill et al., 2006).

Auxins and Cytokinins are the most widely used plant growth regulators in plant tissue culture and usually are used in combination (Zebarjadi et al., 2008; Ghasempour et al., 2012). Result of some studies revealed that auxins played an important role in the callus induction (Baskaran et al., 2006; Ghasempour et al., 2012). Furthermore, they showed that

cytokinins facilitated the effect of auxins in callus induction (Ghasempour et al., 2012; Soheilikhah et al., 2013). Also, the combination of BAP and NAA increased the percentage of organogenesis and the development of the explants (Soheilikhah et al., 2013).

Therefore, successful utilization of plant biotechnology for plant improvement requires the development of an efficient shoot regeneration system from cultured cells or tissues. The development of an efficient micropropagation protocol can highly support breeding of this potential and adaptive oil crop and the establishment of cell culture has considerable potential in the future as an alternative for the production of new secondary metabolites (Stockigt et al., 1995; Ghasempour et al., 2007; Ghasempour et al., 2012).

Development of *in vitro* shoot regeneration techniques is essential for introgression of desirable traits from alien sources (Radhika et al., 2006). Gene transfer techniques are currently being used for genetic modification of agronomically important characters and also the structure and composition of fatty acids in oilseed yielding crops (Ghasempour et al., 2012). An efficient *in vitro* plant regeneration system is a basic necessity for such approaches.

Recently, there is a great demand for the use of plant-based medicaments in place of synthetic drugs. As a result of non-scientific exploitation, most of the medicinal plant resources are being threatened and are on the verge of extinction. Therefore, application of this technology provides the raw materials required for the isolation of drugs by the pharmaceutical industries without depending on natural plant resources (Hanumanaika and Venkatarangaiah, 2008; Ghasempour et al., 2012; Soheilikhah et al., 2013).

In addition, the establishment of cell and tissue culture has a considerable potential in future as an alternative to traditional agriculture for the production of known and new secondary metabolites (Ghasempour et al., 2007; Ghasempour et al., 2012). The production is free from all the disadvantages of whole crop and medicinal plants field cultivation. This technology can provide a continuous and constant year-



Fig. II. Shoot regeneration from callus MS medium containing 1.0 mg l^{-1} BAP + 0.1 mg l^{-1} NAA

round supply of natural plant products, which may be more easily purified. Methods for the production of important components from plant cells have been developed in safflower, such as α -tocopherol (Ghasempour et al., 2012; Soheilikhah et al., 2013), although further research is necessary to improve their economic efficiency.

The present study showed the importance of biotechnological interference in this plant to overcome plant-to-plant variability in their active constituents. This study aimed to

develop a standard protocol to initiate multiple shoot culture of plant that may provide a good source of pharmacologically active plant constituents.

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