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In vitro Callus Induction and Shoot Regeneration in Hollyhocks (*Althaea digitata*)

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ABSTRACT

Althaea digitata (Boiss.) is considered as one of the valuable medicinal plants in the world which is belong to Malvaceae family. Tissue culture techniques have been extensively used for improving and studying of traits in medicinal plants. The present study was conducted to induce callus from shoot, root and leaf explants and shoot regeneration in *A. digitata*. Explants were cultured on Murashige and Skoog (MS) medium supplement with different concentrations and combinations of 6-benzylaminopurine [BAP] (0, 1, 3, and 5 mg L^{-1}), Kinetin [KIN] (0.1 mg L^{-1}), naphthalene acetic acid [NAA] (0.1 mg L^{-1}) and 2,4-dichlorophenoxyacetic acid [2,4-D] (0, 2, 5 and 10 mg L^{-1}). Best results for callus induction was observed in shoot tip explants on MS+5.0 mg L^{-1} 2, 4-D+0.1 mg L^{-1}, KIN (82.98%). After callus initiation, it was immediately transferred to MS medium containing BAP and NAA. Incubation for about 10-12 weeks on the same culture medium resulted shoot buds initiation from the callus. The Maximum regeneration frequency was observed on MS medium containing 0.1 mg L^{-1} NAA and 1.0 mg L^{-1} BAP. The method can be applied in transformation experiments.

1. Introduction

Medicinal plants have been used since ancient times for the treatment of different diseases (Ul Hassan et al., 2014). Despite the emergence of new chemical drugs in contemporary medicine, the application of herbal remedies has not yet been declined (Zargaran et al., 2012). Nearly 80% of the population of developing countries still uses traditional medicine for health care. Modern pharmacopoeias contain a minimum 25% of drugs derived from plants (Kim, 2005).

The genus of Althaea represented worldwide by about 12 species (Hutchinson, 1973; Heywood, 1978) and is distributed throughout Europe (except the north part), North America, North Africa, Caucasus and southern Russia, and from Anatolia to Afghanistan (Hutchinson, 1964; Nasır and Ali, 1979; Heywood, 1978). This genus shows the highest counts of species in Russia, Iran, and Turkey (Iljin, 1949; Nasır and Ali, 1979). It is a perennial herb belonged to family Malvaceae which includes 80 genera and about 1000 species in the world. They are grow throughout the world except the cold regions (Uzunhisarcikli and Vural, 2012).

A. digitata (Boiss.) is a traditional Persian medicine which has antitussive, antioxidant, expectorant, anti-inflammatory, antimicrobial, and laxative therapeutic properties (Ameri et al., 2015). For example, this herb can decrease the grade of dry mouth in patients who received radiotherapy due to cancer in the head and neck area (Ameri et al., 2016).

Primary chemical constituents of Althaea include mucilage, polysaccharides, flavonoids (quercetin, kaaempferol), asparagines, tannins, lecithin, and pectin. The whole plant, particularly the root, abounds with a mild mucilage, which is emollient (Faccila, 1990). Advanced biotechnological methods of culturing plant cells and tissues should provide new means of conserving and ra-

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pidly propagating valuable, rare, and endangered medicinal plants (Nalawade et al., 2003). Tissue culture is an age-old practice for in vitro regeneration of plants, especially the medicinally valuable plant or plants that are difficult to propagate in natural environment (Atal and Kapur, 1982).

In recent years, interest to in vitro culture techniques has been increased, which offer a viable tool for mass multiplication and germplasm conservation of rare, endangered, and threatened medicinal plants (Li et al., 2004; Emma et al., 2005). Reliable proliferation of callus and subsequent plant regeneration are important for massive plant propagation, studies on genetic transformation and development of transgenic plants with new trails. The improvement of the micropropagation system for this species could be important for its commercial applicability (Vargas et al., 2004). Production of calli form pieces of steams, leaves and roots is used for determining the culture conditions required by the explants to survive, grow and study cell development (Sen et al., 2014). processes of plant growth require the action and cross talk of phytohormones such as auxins and cytokinins (Bajguz and Piotrowska, 2009). By setting phytohormone concentration in the medium, differences in amount, rate and growth patterns of explants can be observed (Pierik, 1987).

Although plant regeneration has been obtained from tissue cultures of a wide variety of plant species, many members of the Malvaceae have been rather recalcitrant (Mangat and Roy, 1986). So far, many studies were performed on in vitro callus induction in different plants.

For instance, Taimori et al. (2016) were studied the effects of light conditions, different kinds and concentrations of auxins (NAA and 2,4-D) with cytokinin (Kin) in MS medium on callus induction and embryogenesis in *Crataegus pseudoheterophylla*, *C. aronia* and *C. meyeri*. Ghasempour et al. (2014) used the different concentrations of BAP, kinetin, NAA, and 2, 4-D growth regulator hormones in order to in vitro callus induction and shoot regeneration in Safflower (*Carthamus tinctorius* L.).

Pakseresht et al. (2016) evaluated the effects of explants types, plants growth regulators and elicitors on callus induction and cell culture conditions in *Hyssopus officinalis* L. Zinhari et al. (2016) in order to callus induction and direct shoot regeneration in *Lepidium draba* L. studied cotyledon, stem and root explants in MS medium with different concentrations of NAA, BAP and 2,4-D in two separate tests.

Soorni and Kahrizi (2015) carried out an experiment for the evaluation of callus induction optimization in cumin on MS medium supplemented with different concentrations of 2,4-D (0, 0.5, 1 and 2 mg L^{-1}) plus 0.1 mg L^{-1} kinetin in different explants (root, shoot, leaf, embryo and seed) of cumin accessions.

Jafari et al. (2016) in order to find the best medium composition for callus induction in *Mentha pulegium*, two explants (leaf and hypocotyl) of *M. pulegium* cultured on MS medium supplemented with BA, NAA and 2,4-D.

So far, there is no report on in vitro method of tissue culture for *A. digitata* (Boiss.) in order to improve its cultivation. Therefor this first report on in vitro multiplication of this plant through direct plant regeneration technique offers an effective alternative method of propagation for this important multipurpose medicinal plant. The present paper reports the establishment of callus cultures in *A. digitata* and evaluation of basal media and growth regulator requirements necessary to maximize and sustain continuous growth of callus as well high frequency regeneration of shoots.

2. Materials and Methods

2.1. Plant material

The seeds of wild plants of *A. digitata* were collected from grasslands near of Kermanshah (Latitude: 34° 18'N, longitude: 047°30'E at an elevation of 1374 m above sea level), in the west part of Iran. After removal of the seed coat, seeds were sterilized in 2% (v/v) sodium hypochlorite for 20 minute and washed (5 changes) with sterile distilled water to germinate in 150 ml Erlenmeyer flasks containing 30 ml of full-strength MS salts (Murashige and Skoog, 1962) and 0.7% (w/v) agar at pH 5.7 All the cultures were maintained in growth chamber with 16-h photoperiod at 25±1c° for 14 days.

2.2. Callus induction and subculture

In order to test the effects of intended materials on callus formation, NAA and 2,4-D as auxins and 6-BA and kinetin as cytokinins were added in to MS medium. The range of concentrations of phytohormone varied from 0.1-10 mg L^{-1}. Each

experiment contained at least 25 replicates and repeated three times. Data were recorded after 5 weeks from culture initiation and then well grown callus induced from explants were selected to transfer to MS medium for subculture.

2.3. Callus initiation

The sterilized leaf, shoots and roots explants from 10-15 day old seedlings were divided to 0.5-1.0 cm pieces and cultured on agar solidified MS medium solidified medium supplemented with different concentrations and combinations of 2,4-dichlorophenoxyacetic acid (2,4-D) and kinetin in order to callus formation. The cultures were transferred to the same medium every 15 days. After two times of subculture, the growing states of callus were compared.

2.4. Shoot regeneration from Callus

The callus obtained from various explants was used for regeneration on MS medium supplemented with different concentrations and combinations of BAP and NAA. The regeneration medium contained full the MS contents + 0,1,3,5 mg L^{-1} benzyl adenine (BA) and 0.1 mg L^{-1} NAA. The frequencies of the callus induction and regeneration were determined as the percentage of explants producing callus and the percentage of calli producing fully regenerated plants, respectively.

CIF (%) = No. of explants producing callus/ No. of explants plated * 100

PRF (%) = No. of calli regenerated plantlets/ No. of calli plated for regeneration * 100

Where:

CIF = callus induction frequency

PRF = plant Regeneration Frequency

3. Results and Discussion

At first, leaf, shoot tip and roots explants were cultured on MS medium with different concentrations of 2,4-D and KN (0.1 mg L^{-1}) for callusing. The highest result in term of percentage response (82.98%) and nature of the callus were obtained with the combination of 2,4-D (5 mg L^{-1}) and KIN (0.1 mg L^{-1}) for shoot tip explants after 25-30 days (Table 1, Fig.1a).

Table1. Callus induction from shoot, root, and leaf segments of *A. digitata* (Boiss.) grown on MS medium supplemented with various concentrations of 2,4-d and kin after 25-30 d of culture

	Plant Growth Regulators Concentrations (mg L^{-1})		
Explant	2,4-D	KIN	(Mean ± SE)
Shoot Segments	0	0.1	20.19^{f} ± 0.23
	2	0.1	34.94^{e} ± 0.14
	5	0.1	82.98^{a} ± 0.05
	10	0.1	49.80^{cd} ± 0.09
Root Segments	0	0.1	0 ± 0.0^{h}
	2	0.1	33.81^{e}± 0.14
	5	0.1	72.50^{b} ± 0.0
	10	0.1	57.65^{c} ± 0.08
Leaf Segments	0	0.1	10.20^{g} ± 0.47
	2	0.1	27.75^{ef} ± 0.17
	5	0.1	44.62^{d} ± 0.1
	10	0.1	81.39^{a} ± 0.06

About 81.39% of the leaf-explants successfully produced calli with the combination of 2,4-D (10 mg L^{-1}) and KIN (0.1 mg L^{-1}) after 25-30 days (Fig. 1c). The percentage response of root explants was low in compare to the leaf and shoot tip explants. The highest callusing (72.50%) for root explants, was observed with the combination of 2,4-D (5 mg L^{-1}) and KIN (0.1 mg L^{-1}) after 20-25 days (Fig. 1b). The significant differences were observed between explants, hormone concentration and their interactions for callus induction (Table 2).

According to Soorni and Kahrizi (2015) the accession, explant, accession × explant and 2,4-D × explant interactions had statistically significant effects ($P<0.01$) on callus induction percentage and callus growth rate traits in Cumin (*Cuminum cyminum* L.).

Table 2. Analysis of variance (ANOVA) based on factorial experiment for callus induction

Source of Variation	df	Mean Square
Explant (A)	2	143.476**
Hormone (B)	3	6471.823**
A× B	6	732.677**
Error	24	23.201
CV%	11.21	

* and **: Significant at the 5% and 1% probability levels, respectively.

The shoots were initiated from the callus obtained from all the three explants cultured on MS medium with different concentrations of BAP and NAA, alone or in combination. The best shooting (20.33%) was observed with MS medium supplemented with BAP (1 mg L^{-1}) and NAA (0.1 mg L^{-1}) after 10-12 weeks (Fig. 1d and e), which was used for further regeneration and transformation experiments (Table 3).

Table3. Shoot regeneration from callus of *A. digitata* (Boiss.) on MS medium supplemented with various plant growth regulators after 10-12 week of culture

Plant Growth Regulators (mg L^{-1})		Cultures Producing Shoots (%)
BAP	NAA	(Mean ± SE)
0	0.1	0^{c} ± 0.0
1	0.1	20.33^{a} ± 0.08
3	0.1	12.66^{b} ± 0.14
5	0.1	0^{c} ± 0.0

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

Multiple shoot culture of medicinal plants is a good source of plant active constituents (Eisenbeiss et al., 1999; Shrivastava and Dubey, 2007).

The concentration of cytokinin used significantly affected the percentage shoot regeneration, shoot numbers and shoot length (Bohidar et al., 2008). In our experiment lower concentrations though more number of buds were observed, the growth of the shoots was stunted and basal callusing of the shoots was noticed (Table 4). Cytokinin concentration has been reported to be decisive for shoot proliferation and elongation of many medicinal plant species (Rout et al., 2000; Mohapatra and Rout, 2004). Various factors affecting on induction, maintenance, and regeneration of somatic embryos have been established (Fitch and Moore, 1993; Aftab and Iqbal, 1999). But these studies have followed the classical two steps method i.e., production of callus in the first step using high auxin (2,4-D) and low cytokinin (KIN or BAP) and in the second step shoot regeneration was obtained using medium containing very low or no auxin and inclusion of cytokinins. 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 used together (Gang et al., 2003). The result revealed that auxins play an important role in the callus induction (Skoog and Armstrong., 1970) and different types of auxins had various effects (Baskaran et al., 2006). Furthermore, the cytokinins facilitated the effect of auxins in callus induction (Rao et al., 2006; Yang et al., 2008). The combination of BAP and NAA increased the percentage of organogenesis and the development of the explant (Arous et al., 2001).Zinhari et al. (2016) in study of callus induction and direct shoot regeneration in *L. draba* L. explants, using of different concentrations of growth regulators NAA and BAP, reported that 1 mg LP-1P BAP without NAA induced the highest direct shoot generation in the root explants *L. draba* L. Cotyledons explants in 2 mg L^{-1} NAA and 0.5 mg L^{-1} BAP produced the highest root production rate and the highest root generation.

Table 4. Analysis of variance (ANOVA) based on CRD experiment for shoot regeneration

Source of Variation	df	Mean Square
Calluses	3	301.639**
Error	8	3.167
CV(%)	21.57	

* and **: Significant at the 5% and 1% probability levels, respectively.

According to Jafari et al. (2016) the hormone levels of BA free and 1 mg L^{-1} NAA (86.00%), 0.5 mg L^{-1} BA and 0.5 mg L^{-1} NAA (86.00%), 0.5 mg L^{-1} BA and 1 mg L^{-1} NAA (92.00%) and 1 mg L^{-1} BA and 2 mg L^{-1} NAA (88.00%) had the highest effects on callus induction percentage in pennyroyal (*M. pulegium* L.). Tissue culture technology offers an alternative method for the conservation of germplasm as well as micro propagation of medicinally important plant resources.

Presently there is 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 depleting natural plant resources (Hanumanaika and Venkatarangaiah, 2008).

a b c d e

Fig. 1. Callus induction and shoot regeneration in *A. digitata* (Boiss.). a: callus induction from shoot segment on MS medium containing 5mg L^{-1} 2,4-D + 0.1mg L^{-1} KIN. b: callus induction from root segment on MS medium containing 5mg L^{-1} 2,4-D + 0.1mg L^{-1} KIN. c: callus induction from leaf segment on MS medium containing 10 mg L^{-1} 2,4-D + 0.1 mg L^{-1} Kin. d and e: shoot regeneration from callus MS medium containing 1.0 mg L^{-1} BAP + 0.1 mg L^{-1} NAA.

The present study showed the importance of biotechnological interference in *A. digitata*, to overcome plant-to-plant variability in their active constituents. This study aims 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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