J. Agr. Sci. Tech. (2017) Vol. 19: 1429-1435

RESEARCH NOTES

Thin Cell Layer, a Suitable Explant for *In vitro* Regeneration of Saffron (*Crocus sativus* L.)

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ABSTRACT

Saffron (*Crocus sativus* L.) is a sterile species and biotechnological method is suggested to improve different characteristic in this valuable plant. In this study, an efficient protocol was provided for callus induction and regeneration of saffron using thin cell layer explants. Longitudinally and transversally, thin cell layer explants with approximately 1 mm thickness of apical buds were cultured on MS medium supplemented with different concentrations of 2,4-Dichlorophenoxyacetic acid (2,4-D), 6-BenzylAminoPurine (BAP) and 1-NaphthaleneAcetic Acid (NAA). The highest amount of callus induction (100%) was obtained from transverse thin cell layer explants of apical bud in MS medium containing 2 mg L^{-1} BAP and 2 mg L^{-1} NAA during 3 months incubation under dark condition at 20°C. The maximum percent of shoot regeneration (75%) was observed on the MS medium containing 0.5 mg L^{-1} BAP. The results of this investigation revealed that the thin cell layers from buds are suitable explants for regeneration.

Keywords: Callus induction, Tissue culture, Growth Regulators, Shoot induction.

INTRODUCTION

Saffron (*Crocus sativus* L.) belongs to the Iridaceae family and is cultivated for its three lobate red stigmas that are characterized as the world's most valuable spice by weight (Melnyk *et al*., 2010). Having more than 90% of the world total production makes Iran as one of the leading countries in saffron production with 47,200 ha cultivated area and 170 tons annual production (Emadi and Saiedirad, 2011). Dried stigmas have been used in medicine, food seasoning, and coloring since centuries. It also has many volatile and nonvolatile components. Crocin, as a main carotenoid compound of saffron, is responsible for color and has remarkable pharmacological effects, such as protection against cardiovascular diseases and inhibition of

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[DOR: 20.1001.1.16807073.2017.19.6.16.1]

cancer cell development (Abdullaev, 2002). Due to its triploid nature, saffron is sterile and is not able to produce seed (Sharma *et al*., 2008). Some protocols have been developed for *in vitro* micropropagation of saffron by several researchers (Ting *et al*., 1979; Homes *et al*., 1987; Ilahi *et al*., 1987). Classical breeding of autotriploid plants such as saffron is very difficult (Basker and Neghbi, 1989). Due to several remarkable disadvantages of classical methods, tissue culture and genetic engineering techniques are suggested to improve different characteristic of this plant (Chaloushi *et al*., 2007). In order to have successful results in genetic engineering, high rates of regeneration and low rates of escape are needed. Using explants with large size in gene transformation studies increase the chance of regeneration for both transformed and untransformed cells which leads to chimerism (Teixeira da Silva, 2003). Regarding the advantages of Thin Cell Layer (TCL) method, this method could significantly reduce the possibility of chimerism. The TCL system consists of small size explants of different plant organs, longitudinally (lTCL), or transversally (tTCL), which are different in tissue type (Altamura *et al*., 1993; Tran Thanh Van, 1980). Therefore, they could be used as suitable systems for selecting a specific cell or tissue layer, in order to induce a specific morphogenic programs which leads to an efficient and successful regeneration of transformed tissue (Teixeira da Silva, 2003).

The regeneration of many plant species through TCL method has been reported so far (Altamura *et al*., 1993; Gozu *et al*., 1993; Ozawa *et al*., 1998; Falasca *et al*., 2004; Shinoyama *et al*., 2006; Mirmasoumi *et al*., 2013). TCL has also been successful as an efficient method for enhanced production of secondary metabolites and pharmaceuticals through transgenic organ cultures, such as those produced by *Agrobacterium rhizogenes* (Teixeira da Silva, 2003).

In previous studies of saffron, some protocols were provided for in vitro micropropagation by conventional methods (Sharifi *et al*., 2010; Devi *et al*., 2014). In the present study, an efficient procedure for callus induction, regeneration and cormlet production using TCL explants is introduced.

MATERIALS AND METHODS

Plant Materials and Culture Media

Saffron corms were kept under dark and cold (4°C) conditions for about three months. The apical bud excised from the corms were thoroughly washed under running tap water for 20 minutes and sterilized by dipping in 70% ethanol for 30 seconds followed by sodium hypochlorite (containing chlorine 2% (w/v)) for 15 min and washed with sterile distilled water for 3 times.

Two types of thin cell layers, i.e. longitudinally (lTCL) and transversally (tTCL), with approximately 1 mm thickness from apical bud were used as explants (Figure 1-a). Experiments were done in three steps as follows:

1) Callus induction: tTCL and lTCL of apical buds were cultured in MS culture media supplemented with 0, 0.5, 1, 2 mg L^{-1} 2,4-D or MS media containing different concentrations of BA and NAA (0, 0.5, 1, 2, 5 mg L^{-1}).

2) Shoot regeneration: The induced calli from tTCL explants were transferred to MS media containing 0.05% activated charcoal with the different concentrations of BA (0, 0.5, 1, 2, 5, 10 mg L^{-1}) and NAA (0, 0.5, 1, 2 mg L^{-1}).

3) Root initiation: Shoots were transferred to MS media containing 0, 0.5, 1, 2 mg L^{-1} NAA and 0.05% activated charcoal.

In all experiments, MS media were supplemented with 30 g L^{-1} sucrose and were solidified with 7 g L^{-1} agar-agar (Merck). The pH of all culture media was adjusted to 5.7–5.8 before autoclaving.

Culture Conditions and Statistical Analysis

Explants were incubated in the dark at 25±2°C under a 16:8 hour light/dark photoperiod for callus induction and

[DOR: 20.1001.1.16807073.2017.19.6.16.1]

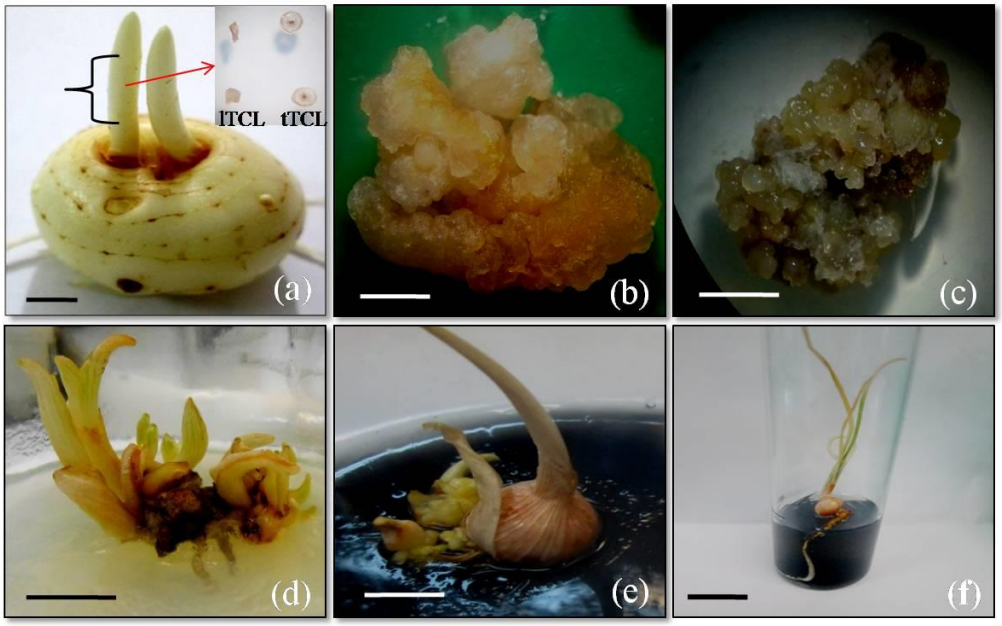

Figure 1. Regeneration of saffron using thin cell layer explant. (a) Two types of thin cell layers, longitudinally (lTCL) and transversally (tTCL) with approximately 1 mm thickness from apical bud used as explants (Bar= 1 cm); brace shows the explant location, arrow shows type of explant. Callus induction on TCL explant from apical bud; (b) At 2 mg L^{-1} 2,4-D (Bar= 0.5 cm); (c) At 2 mg L^{-1} BAP and NAA (Bar= 1.0 cm); (d) Regeneration at 1 mg L^{-1} BAP after 6 weeks (Bar= 2.0 cm); (e) Produced cormlet after 5 months at 1 mg L^{-1} BAP (Bar = 1.5 cm), and (f) Rooting of cormlet at 2 mg L^{-1} NAA (bar = 1.5 cm)**.**

regeneration. The experiments were performed in factorial arrangement based on Completely Randomized Design (CRD) with four replications and five explants per replication. All means were compared using Duncan's multiple range tests at the 5% probability level ($P \leq 0.05$). All computations were made using the SAS and SPSS statistical analysis package.

RESULTS

Effect of 2,4-D on Callus Induction from TCL Explants of Apical Buds

The first calli on TCL explants were observed at the end of the second month. They were mainly golden and yellow in color. The results showed that 2,4-D had a significant effect on callus induction and the higher rates of callus formation were observed at high concentrations of 2,4-D in both tTCL and lTCL explants. In media without hormone or supplemented with 0.5 mg L^{-1} 2,4-D, no callus induction was observed in lTCL explants. While, in the tTCL explants, the low rate (10%) of callus formation was observed at those media. The frequency of callus induction increased with increasing the concentration of 2,4-D. The longitudinal explants induced callus only at 1 and 2 mg L^{-1} 2,4-D, while in the transversal explants, the callus was formed at all four levels of the 2,4-D concentration. The highest rate of callus induction (90%) was observed from tTCL explants on the medium containing 2 mg L^{-1} 2,4-D (Figure 1-b), while at the same concentration, only 15% callus induction was obtained on lTCL explants (Table 1).

Effect of BAP and NAA on Callus Induction in TCL Explants of Apical Buds

[DOR: 20.1001.1.16807073.2017.19.6.16.1]

Table 1. Effect of explant type and 2,4-D concentration on callus induction of TCL apical buds explants in saffron

Growth regulator (mg L^{-1})	Callus induction (%)	
2,4-D	lTCL	tTCL
0.0	0^{b}	10^{b}
0.5	0^{b}	10^{b}
1.0	5^{b}	10^{b}
2.0	15^{b}	90^{a}

The longitudinal and transversal explants showed high similarities in terms of quality and the shape of callus. The first sign of callus formation was observed with changing the color of explants in the third week. Then, the central parts of explants became swollen and callus formed after two weeks. In total, after 5 weeks of culture, callus induction was occurred. In the tTCL explants, the induced calli were generally crisp, clear, and white to yellow and some of the calli turned to spherical calli after the second subculture. The results showed that response of tTCL and lTCL explants on various NAA and BAP concentrations were significantly different ($P \leq 0.05$). Low rate of callus induction was observed in medium without growth regulators in tTCL explant. The highest rate of callus induction was obtained in medium containing 2 mg L^{-1} NAA and BAP in both tTCL (Figure 1-c) and lTCL explants (Table 2).

Table 2. Effect of NAA and BAP on callus induction of lTCL and tTCL apical buds explants in saffron.

Growth regulators (mg L^{-1})		Callus induction (%)	
NAA	BA	lTCL	tTCL
0	0	0^{g}	5^{e}
0	0.5	30^{defg}	5^{e}
0	1	25^{efg}	5^{e}
0	2	50^{cde}	10^{e}
0.5	0	5^{g}	5^{e}
0.5	0.5	25^{efg}	5^{e}
0.5	1	50^{cde}	85^{ab}
0.5	2	15^{fg}	35^{d}
1	0	45^{cdef}	90^{a}
1	0.5	60^{bcd}	55^{cd}
1	1	85^{ab}	65^{bc}
1	2	30^{defg}	85^{ab}
2	0	20^{efg}	100^{a}
2	0.5	65^{bc}	55^{cd}
2	1	20^{efg}	35^{d}
2	2	95^{a}	100^{a}

Effect of BAP and NAA on Regeneration and Rooting

Various combinations of NAA and BAP had significant effects on the regeneration of calli induced from tTCL explants. The maximum percent of regenerations (75 and 65%) was observed in the media containing 0.5 and 1 mg L^{-1} BAP, respectively (Table 3). Our results showed that to obtain high

Table 3. Effect of BA and NAA on regeneration of saffron from TCL explants.

Growth regulators (mg L^{-1})		Regeneration (%) [a]
BA	NAA	
0	0	5^{ef}
0	0.5	5^{ef}
0	1	0^{f}
0	2	0^{f}
0.5	0	75^{a}
0.5	0.5	5^{ef}
0.5	1	0^{f}
0.5	2	5^{ef}
1	0	65^{ab}
1	0.5	$15d^{ef}$
1	1	5^{ef}
1	2	0^{f}
2	0	25^{cd}
2	0.5	30^{cde}
2	1	20^{ef}
2	2	$5d^{ef}$
5	0	$10d^{ef}$
5	0.5	$10d^{ef}$
5	1	10^{def}
5	2	0^{f}
10	0	45^{bc}
10	0.5	10^{def}
10	1	0^{f}
10	2	0^{f}

[a] Data collected from both lTCL and tTCL apical buds explants.

[DOR: 20.1001.1.16807073.2017.19.6.16.1]

rates of regeneration, media without NAA with different levels of BA were more effective than the combination of both NAA and BA. However, among the tested levels of BA, the low levels represented the most inducible concentrations. The maximum number of shoots per explant (4.8) was observed in the medium containing 2 mg L^{-1} BAP (data not shown). MS Medium consisting of 2 mg L^{-1} NAA showed 35% rooting (35%) (data not shown).

Shoots produced in the regeneration stage were cultured on the medium containing 1 mg L^{-1} BAP (Figure 1-d) and they produced small cormlets after 6 weeks. These cormlets were transferred to the medium containing 60 g L^{-1} of sucrose, 1 g L^{-1} of activated charcoal and 1 mg L^{-1} BAP for more growth. The average weight of cormlets was 1.3 g after three subcultures (3 weeks interval). The cormlets were natural in terms of appearance as well as the growth potential and produced apical buds, leaves, and roots in *in vitro* condition at 18°C (Figures 1-e and -f).

DISCUSSION

In the present experiment, the highest rates of callus induction was observed in the media containing 2,4-D from tTCL explants. However, in the media containing NAA and BA, both explants tTCL and lTCL showed high rates of callus induction (100 and 95%, respectively). The highest regeneration rate using TCL explants (75%) was found in the medium containing 0.5 mg L^{-1} BAP, whilst in the media containing high levels of auxin, the regeneration did not occur.

Our results indicated a negative effect of auxin and a significant positive effect of cytokinin on the regeneration rate. The buds, leaf-like structures, and micro corms formed on the media with different levels of BAP and the decline in shoot regeneration rate was identified with increase in BAP concentration. Igarashi and Yuasa (1994) have reported the 50% regeneration at low levels of NAA and high levels of BAP in saffron. Singh *et al*. (2012) found the highest shoot multiplication (100%) in the medium containing 13.2 µM BAP and 4.6 µM Kin in *Eclipta alba* through tTCL nodal explants. MS medium with half-strength macronutrients and 2% sucrose, supplemented with 1.2 mg L^{-1} NAA and 1.2 mg L^{-1} 6-BA, was optimal for high frequency of shoot regeneration (92%) through tTCL explant in *Dendrobium candidum* (Zhao *et al*., 2007).

Zeybek *et al*. (2012) reported that the production rates of shoots, roots, and corms in saffron were 19, 64, and 33%, respectively. The highest rate of callus initiation was observed on the medium containing 0.25 mg L^{-1} 2,4-D and 1 mg L^{-1} BAP, whilst the highest rate of adventitious shoot was observed in the medium containing 1.5 mg L^{-1} BAP. In the media supplemented with 0.25 mg L^{-1} BAP, regeneration of calli induced from corm explants of saffron was very low and callus production percentage did not exceed 5% (Simona *et al*., 2013). Sharifi *et al*. (2010) reported the highest rate of direct shoot induction from saffron corm explants was obtained on MS medium containing 4.54 µM TDZ.

The TCL explants have been introduced as superior explants compared to other conventional explants for plant regeneration (Nhut *et al*., 2003; Rout *et al*., 2006; Singh *et al*., 2009). The present study was undertaken to achieve a high frequency regeneration protocol for *C. Sativus* by applying TCL method. To the best of our knowledge, the present study is the first report to evaluate regeneration from the thin cell layer explant system of *C. sativus.* Our results indicated that TCL explants could be used as efficient regeneration system in saffron which is very helpful for genetic engineering and polyploidy studies of saffron.

ACKNOWLEDGEMENTS

This research was supported by Novin Giti Gene Company (www.ngene.ir), in Incubator center of National Institute of

[DOR: 20.1001.1.16807073.2017.19.6.16.1]

Genetic Engineering and Biotechnology of Iran.

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لایه نازک سلولی ریزنمونه ای مناسب برای باززایی درون شیشه ای زعفران (*Crocus sativus* L.)

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چکیده

گیاه زعفران (*Crocus sativus* L.) گیاهی عقیم بوده و بنابراین استفاده از روشهای بیوتکنولوژی برای اصلاح این گیاه ارزشمند ضروری است. در این مطالعه یک دستورالعمل با کارایی بالا برای تولید کالوس و باززایی زعفران با استفاده از ریزنمونه لایه نازک سلولی ارایه شده است. ریزنمونه های لایه نازک سلولی طولی و عرضی با قطر حدود یک میلیمتر از جوانه های انتهایی جدا شدند و بر روی محیط MS حاوی غلظتهای مختلف 2,4-D، BAP و NAA کشت شدند. بیشترین درصد کالوس زایی (۱۰۰٪) از ریزنمونه های لایه نازک سلولی در محیط MS حاوی ۲ میلی گرم در لیتر BAP و ۲ میلی گرم در لیتر NAA در طول ۳ ماه نگهداری در شرایط تاریکی و در دمای ۲۰ درجه سانتیگراد بدست آمد. بیشترین درصد تشکیل شاخساره زایی (۷۵٪) در محیط MS حاوی ۰/۵ میلی گرم در لیتر BAP مشاهده شد. نتایج این تحقیق نشان داد که لایه نازک سلولی حاصل از جوانه، ریزنمونه مناسبی برای باززایی می باشد.

[DOR: 20.1001.1.16807073.2017.19.6.16.1]