JOURNAL OF HORTICULTURE AND POSTHARVEST RESEARCH
2021, VOL. 4(SPECIAL ISSUE: RECENT ADVANCES IN SAFFRON), 43-56

Journal of Horticulture and Postharvest Research

Journal homepage: www.jhpr.birjand.ac.ir

University of Birjand

The effect of developmental stages of corm, type of medium and plant growth regulators in callus induction of *Crocus sativus* L.

Seyed Mahdi Ziaratnia[1,*] and Somaye Amini[2]

1, *Department of Food Biotechnology, Research Institute of Food Science and Technology (RIFST), Mashhad, Iran*
2, *Department of Horticultural Sciences, Gorgan University of Agricultural Sciences and Natural Resources, Gorgan, Iran*

ARTICLE INFO

Original Article

Article history:
Received 5 December 2020
Revised 25 February 2021
Accepted 21 April 2021
Available online 2 June 2021

Keywords:
Callogenesis
Culture Medium
Developmental Stage
Plant growth regulators
Saffron

DOI: 10.22077/jhpr.2021.3684.1166
P-ISSN: 2588-4883
E-ISSN: 2588-6169

***Corresponding author:**
Department of Food Biotechnology, Research Institute of Food Science and Technology (RIFST), Mashhad, Iran.

Email: m.ziaratnia@rifst.ac.ir

ABSTRACT

Purpose: The efficiency of callus induction as source of bioactive compound is a critical step in the cell suspension culture for commercial production of important secondary metabolites. One of the main factors affecting callus efficiency is the developmental stage of explants. On the other hands, it has been declared that the optimization of medium composition has a significant influence on callogenesis and increment of biomass. **Research method:** The aim of this study was the evaluation of different developmental stages of saffron corms as a source of explant preparation (immature and mature corms), plant growth regulators (PGRs) combinations (1-Naphthaleneacetic acid (NAA); 2, 4-dichlorophenoxyacetic acid (2,4-D); 6-Benzyladenine (BA) and kinetin (Kin)) and type of medium (Gamborg (B_5) medium and Murashige and Skoog (MS) medium) in callus induction in saffron corms to increase cell biomass. The media were supplemented with different combinations of 1-Naphthaleneacetic acid or 2, 4-dichlorophenoxyacetic acid (2, 4, 8 mgL^{-1}) as auxins and 6-Benzyladenine or kinetin (1, 4, 8 mgL^{-1}) as cytokinin. **Findings:** The results showed that mature corms harvested in May had the best developmental stage for callogenesis. The maximum callus formation was recorded in B_5 medium supplemented with 2,4-dichlorophenoxyacetic acid (4 mgL^{-1}) and kinetin (1 mgL^{-1}) with 2.61 g fresh weight. **Limitations:** No limitations to report. **Originality/Value:** This protocol for sampling explant and callus formation was found to make suitable sources of plant material for further study in production of bioactive compounds via cell suspension cultures.

INTRODUCTION

Crocus sativus L., is an herbaceous perennial plant species (Botanically saffron is an annual plants but in agronomy is usually cultivated as perennial) of Iridaceae family that has been spread out in Mediterranean and west of Asia (Verma et al., 2016; Fallahi & Mahmoodi, 2018). This autumn flowering plant is an auto-triploid form of a species found in eastern Greece. The origin of saffron is proposed to be from western or center of Asia (possibly Iran) (Mathew, 1977). Saffron is the world known appreciated exceptional natural food additive with valuable medicinal properties. Stigmas as the main part of saffron are composed of chemical components such as colored carotenoids, crocin, crocetin and monoterpene aldehydes, picrocrocin and safranal (Lozano et al., 2000). Although saffron potentially is very valuable and applicable in several industries including medicine, food, health, cosmetic and perfume, it has limited use due to the high price (Mzabri et al., 2019).

Saffron production is extremely laborious which is due to the time-consuming process of manual picking up of flowers, separating stigmas (Halim et al., 2018) as well as very limited harvesting time. All these reasons have still caused it to be the most expensive spice in the world. It is also reported that to make 1 kg saffron dried stigma, around 150,000–200,000 flowers should be harvested, which takes over 400 h of hand-labor (Plessner et al., 1989).

Plant cell, tissue culture techniques offer a great potential for mass propagation of organs (Devi et al., 2014) or large-scale of plant cells in a cell suspension culture system for production of valuable compounds (Georgiev et al., 2009). The successful application of plant cell culture techniques depends on the availability of an efficient system or protocol for callus induction (Xu et al., 2009). On the other hand, callus and cell culture have also been reported to produce some active ingredients and specific medicinal compounds (Veraplakorn 2016). To reach that purpose, several factors such as medium composition, genotype, explants source, and environmental conditions can influence callus induction (Abbas et al., 2018). Medium compositions, type and concentrations of plant growth regulators as well as explants are the most important factors affecting plants tissue culture (Sajjadi & Pazhouhandeh 2015).

Saffron tissue culture has a history more than 30 years with several research results reported in this field (Husaini et al., 2010). Callus induction on saffron corm has also been described by several researchers (Dahr & Sapru 1993; Karaoğlu et al., 2006; Sharma et al., 2008; Blazquez et al., 2009; Georgiev et al., 2009). The first successful case of saffron tissue culture using corm explant was reported by Ting et al. (1979). There are also many reports in saffron cell, tissue and organ cultures regarding optimization of medium using different types and medium compositions as well as PGRs combinations. For instance, Safarnejad et al. (2016) reported the maximum callus induction was achieved on MS medium supplemented with 1 mgL^{-1} 2,4-D + 2 mgL^{-1} BAP. In case of *in-vitro* direct and indirect saffron organogenesis, it was reported that a combination of 2,4-D (0.25 mg L^{-1}) and BAP (1 mgL^{-1}) was superior for indirect organogenesis (Yildrim, 2007). Apart from the medium optimization many studies have been carried out on type of saffron organs as explants source including leaves (Raja et al., 2006), corms (Milyaeva et al. 1995; Sharifi et al., 2010), full ovary and half ovary (Choob et al., 1994; Mir et al., 2010) due to its influence on callogenesis or organogenesis. In case of saffron, because the availability of flower organs is very short and limited in two months (October to November), they do not look very useful for explant sampling while corms are more available due to their ability to be stored in controlled conditions.

Many researchers have emphasized on critical impact of genotype, physiological age of tissue used as explant specimens on plant callus induction and regeneration (Dayal et al., 2003; Thomas 2003; Sun et al., 2009; González et al., 2012). They believe the different responses of callogenesis may be related to the explants potential which can be attributed to the endogenous

hormones (Mazumdar et al., 2010). The results of some studies demonstrated that younger explants are more susceptible to callogenesis, because they are still developing, less differentiated and consequently have more metabolically active cells (Holme & Petersen 1996; Ibrahim & Debergh, 2001; Murthy & Pyati, 2001; Mazumdar et al., 2010), in some other cases, the results showed that the older explants were more suitable than younger ones on callogenesis efficiency (Antonelli & Druart, 1990). Verma et al. (2016) investigated on callus induction, somatic embryogenesis and plant regeneration in five species of Turkish *Crocus* using three different explants (leaf, stem and corm) cultured on four different media. Their results confirmed that species type, organ and also media with different levels of plant growth regulators can influence on several characteristics, including callus induction (Verma et al., 2016).

Indeed, saffron growing period (June to June) is divided to six principal stages starting from sprouting, cataphylls and flowers appearance, leaves appearance and development, replacement corms development, plant senescence and corm dormancy (Lopez-Corcoles et al., 2015). After flower collecting in October to November, the leaves appear and are followed by corm replacement induction and development until May to June, in which the leaves get yellow and the replacement corms enter the dormancy which is their fully mature stage (Fig. 1).

Despite the fact that many researches have been carried out on saffron callogenesis with different purposes, no attention has been paid to the effect of physiological stage or maturity of saffron corms on the efficiency of callus induction. Considering the medicinal and economic importance of saffron due to the presence of valuable compounds such as crocin, saffron cell suspension cultures have attracted attentions as a very useful method for the production of saffron secondary metabolites in lower costs. Obviously, callus induction is the first and critical step for the establishment of a desired cell suspension system. Therefore, the present study, apart from optimizing the medium compositions by applying different PGRs combinations for the purpose of increasing efficiency of callus induction, for the first time has focused on the comparison of the effect of different physiological and developmental stage of saffron corm callogenesis.

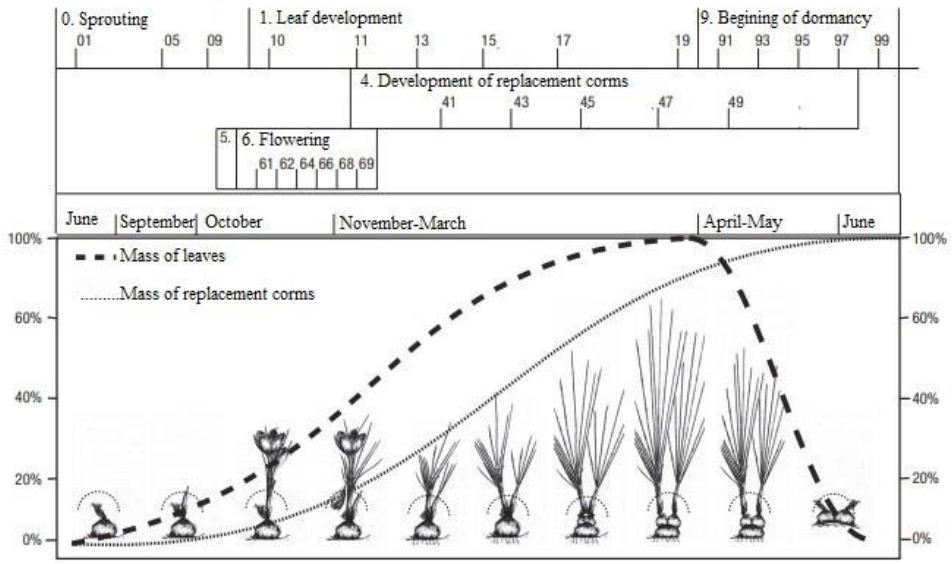

Fig. 1. Scheme of the principal growth stages of saffron (*Crocus sativus* L.) on a monthly basis, (%) (Lopez-Corcoles et al., 2015)

MATERIALS AND METHODS

The present investigation was conducted to develop a protocol for rapid callus induction in saffron. The callus induction potential was assessed on corm explants from different developmental stages in interaction with different media and plant PGRs combinations. Two kinds of corms from different physiological stages were collected in March when the replacements corm are still developing (immature) and in May when the corms are fully developed (mature) used as explant sources. These explants were cultured on two basal media, Gamborg (B_5) (Gamborg et al. 1968) and Murashige and Skoog's (MS) (Murashige and Skoog, 1962), supplemented with various concentrations and combinations of 2,4-dichlorophenoxyacetic acid (2,4-D) or 1-Naphthaleneacetic acid (NAA) with kinetin or benzyl adenine (BA) to find out the best explant source as well as optimum medium type and composition for saffron callogenesis.

Plant material

Saffron corms were collected from an experimental farm at the Research Institute of Food Science and Technology, Khoarsan Razavi Province, Mashhad, Iran, in March and May 2016. After removing the outer covering shells, they were rinsed under running tap water for 45 min. The surface disinfection was done applying 70% ethanol for 2 min then followed by commercial bleach containing 1% sodium hypochlorite for 15 minutes. To remove the disinfectant residues, corms were rinsed three times with double sterile distilled water. Disinfected corms were then cut in several pieces with dimensions of 0.5 – 1.0 cm^2 with a thickness of 1 mm. Corm pieces were included parenchyma, node and internode.

Callus induction

The disinfected explants derived from corm in two developmental stages were then cultured on solid B_5 and MS media containing 3% sucrose with different combinations of Auxins, NAA/2,4-D (2, 4, 8 mgL^{-1}) and Cytokinins, BA/Kin (1, 4, 8 mgL^{-1}). All media were solidified by 0.7% agar then adjusted on pH = 5.7 before autoclaving at 121°C for 20 minutes. Five explants were cultured in each plate (6 cm diameter) at three replicates. Cultures were then incubated at 22±2 °C in the dark conditions.

The interaction of different PGRs combinations and types of explants from two different developmental corms were investigated in MS and B_5 medium. The application of different levels of auxins and cytokinins made 36 PGRs combinations (Table 1). All cultures were sub-cultured in a month intervals. After 2 months, all treatments were compared with each other based on callus fresh weight.

All media component and plant growth regulators (PGRs) were purchased from Sigma, Merck and Fluka companies.

Table 1. Different PGRs treatments in B_5 and MS media

First group	Second group	Third group	Forth group
2,4-D (2mgL^{-1})+Kin (1mgL^{-1})	2,4-D (2mgL^{-1})+BA (1mgL^{-1})	NAA (2mgL^{-1})+Kin (1mgL^{-1})	NAA (2mgL^{-1})+BA (1mgL^{-1})
2,4-D (2mgL^{-1})+Kin (4mgL^{-1})	2,4-D (2mgL^{-1})+BA (4mgL^{-1})	NAA (2mgL^{-1})+Kin (4mgL^{-1})	NAA (2mgL^{-1})+BA (4mgL^{-1})
2,4-D (2mgL^{-1})+Kin (8mgL^{-1})	2,4-D (2mgL^{-1})+BA (8mgL^{-1})	NAA (2mgL^{-1})+Kin (8mgL^{-1})	NAA (2mgL^{-1})+BA (8mgL^{-1})
2,4-D (4mgL^{-1})+Kin (1mgL^{-1})	2,4-D (4mgL^{-1})+BA (1mgL^{-1})	NAA (4mgL^{-1})+Kin (1mgL^{-1})	NAA (4mgL^{-1})+BA (1mgL^{-1})
2,4-D (4mgL^{-1})+Kin (4mgL^{-1})	2,4-D (4mgL^{-1})+BA (4mgL^{-1})	NAA (4mgL^{-1})+Kin (4mgL^{-1})	NAA (4mgL^{-1})+BA (4mgL^{-1})
2,4-D (4mgL^{-1})+Kin (8mgL^{-1})	2,4-D (4mgL^{-1})+BA (8mgL^{-1})	NAA (4mgL^{-1})+Kin (8mgL^{-1})	NAA (4mgL^{-1})+BA (8mgL^{-1})
2,4-D (8mgL^{-1})+Kin (1mgL^{-1})	2,4-D (8mgL^{-1})+BA (1mgL^{-1})	NAA (8mgL^{-1})+Kin (1mgL^{-1})	NAA (8mgL^{-1})+BA (1mgL^{-1})
2,4-D (8mgL^{-1})+Kin (4mgL^{-1})	2,4-D (8mgL^{-1})+BA (4mgL^{-1})	NAA (8mgL^{-1})+Kin (4mgL^{-1})	NAA (8mgL^{-1})+BA (4mgL^{-1})
2,4-D (8mgL^{-1})+Kin (8mgL^{-1})	2,4-D (8mgL^{-1})+BA (8mgL^{-1})	NAA(8mgL^{-1})+Kin (8mgL^{-1})	NAA (8mgL^{-1})+BA (8mgL^{-1})

Table 2. Analysis of variance (ANOVA) of explant age, medium type, PGRs group and PGRs concentration effect on callus fresh weight by factorial design

Source	DF	Seq SS	Adj MS	F value	P value
Explant age (EA)	1	2.46462	2.46462	761.76	0.000
Medium type (MT)	1	18.42228	18.42228	5693.94	0.000
PGRs group (PG)	3	0.04941	0.01647	5.09	0.002
PGRs concentration (PC)	8	1.60845	0.20106	62.14	0.000
(EA)*(MT)	1	0.99092	0.99092	306.27	0.000
(EA)*(PG)	3	0.90666	0.30222	93.41	0.000
(EA)*(PC)	8	0.54190	0.06774	20.94	0.000
(MT)*(PG)	3	0.02797	0.00932	2.88	0.036
(MT)*(PC)	8	1.58623	0.19828	61.28	0.000
(PG)*(PC)	24	13.00518	0.54188	167.48	0.000
(EA)*(MT)*(PG)	3	0.81028	0.27009	83.48	0.000
(EA)*(MT)*(PC)	8	0.68086	0.08511	26.30	0.000
(EA)*(PG)*(PC)	24	2.91285	0.12137	37.51	0.000
(MT)*(PG)*(PC)	24	11.12747	0.46364	143.30	0.000
(EA)*(MT)*(PG)*(PC)	24	3.10120	0.12922	39.94	0.000
Error	288	0.93180	0.00324		
Total	431	59.16808			

Statistical analysis

The layout of the experiment was statistically analyzed in a factorial experiment containing four factors including factor A; developmental stage of corms (immature and mature corm, harvested in March and May, respectively), factor B; two type of basal media (B_5 and MS), factor C; four PGRs groups [(2,4-D+Kin, 2,4-D+BA, NAA+Kin, NAA+BA) and factor D; nine concentration groups for PGRs [Auxin ($2mgL^{-1}$)+Cytokinin ($1\ mgL^{-1}$), Auxin($2\ mgL^{-1}$)+Cytokinin ($4\ mgL^{-1}$), Auxin ($2\ mgL^{-1}$)+Cytokinin ($8\ mgL^{-1}$), Auxin ($4\ mgL^{-1}$)+Cytokinin ($1\ mgL^{-1}$), Auxin ($4\ mgL^{-1}$)+Cytokinin ($4\ mgL^{-1}$), Auxin ($4\ mgL^{-1}$)+Cytokinin ($8\ mgL^{-1}$), Auxin ($8\ mgL^{-1}$)+Cytokinin ($1\ mgL^{-1}$), Auxin ($8\ mgL^{-1}$)+Cytokinin ($4\ mgL^{-1}$), Auxin ($8\ mgL^{-1}$)+Cytokinin ($8\ mgL^{-1}$)] under complete randomized design, with 3 replicates. The recorded data were subjected to statistical analysis by MINITAB software and computing of LSD values to separate means in different statistical groups at the significant level of $p \leq 0.05$.

RESULTS

In this study, the analysis of variance (ANOVA) revealed that the fresh weight of callus was significantly ($p<0.05$) affected by PGRs treatments in different concentrations, the basal medium and developmental stage of the corm (Table 2).

The percentage of callogenesis in March explants demonstrated that induction of callus was started after around 7 days regardless of the PGRs treatments and basal media. The highest percentage of callogenesis (100%) was observed in all treatments after 12 days (data not shown). Although there was no significant difference among the color of induced callus, yellow was the prominent color in all treatments (Fig. 2).

The callogenesis in explants derived from mature corms (harvested in May) started after 4 days with the percentage of 100% in both B_5 and MS medium similar to March explants. It was also found that the corms at different developmental stages can influence on the color of callus, as the color of callus observed in explants derived from mature corms (May) was more reddish than those induced on ones derived from immature corms (Fig. 2).

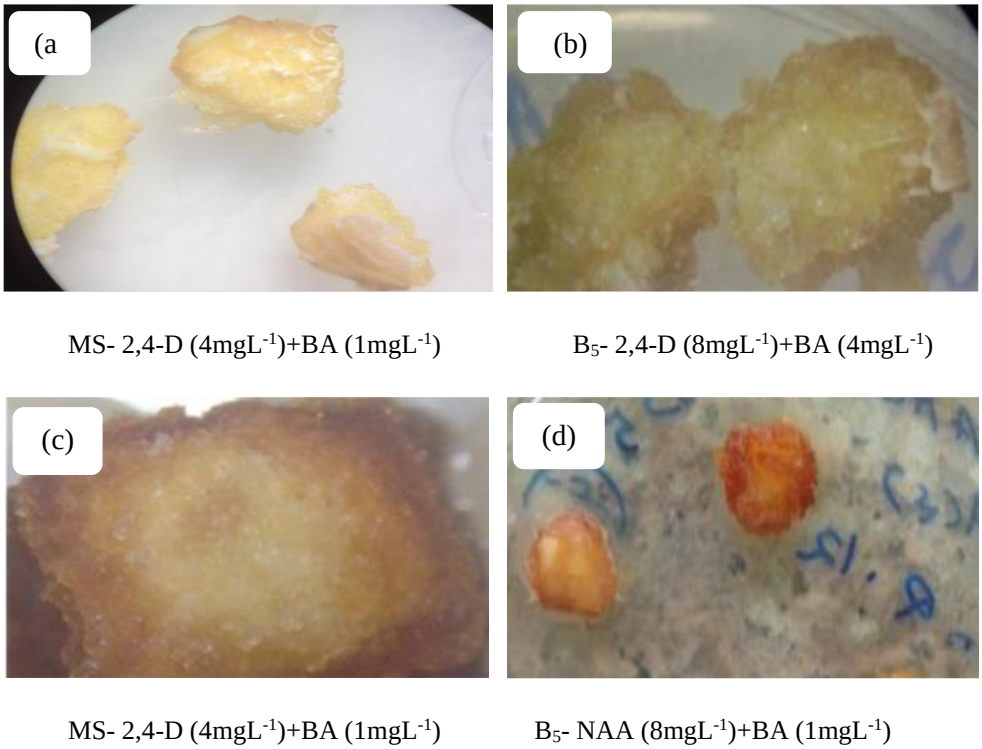

Fig. 2. Comparison of callus color induced on immature explants from corms harvested in March (a, b) and mature explants from corm harvested in May (c, d). There is no difference in the color of calluses formed on immature explant (a and b). While calluses formed on mature explants were different in terms of color (c and d). Red callus was formed only on mature corm (d).

The results also showed that there is a significant interaction effect between corm developmental stages and two basal media when the fresh weight of callus from May derived explants was statistically higher than those prepared from March and in B_5 was also higher than MS medium (Fig. 3). The most striking difference in this response was observed for fresh weight of callus in B_5 in comparison with the MS medium in May which was 0.8 g and 0.29 g respectively. While callogenesis response in March for B_5 medium was reported to be 0.56 g compared to the MS medium with 0.24 g accordingly, it can be said that May is the suitable time for harvesting saffron corms as a source of explants in the point of callus induction view (Fig. 3).

The interaction between corm developmental stages and type of PGRs revealed that the combination of 2,4-D and Kin not only caused the higher callogenesis on May derived explants compared to all of the combinations in March, but also it showed better performance of other PGRs treatments in the same type of explants (May) (Fig. 4). As shown in Figure 4, the best response in callogenesis happened in explants from May with combination of 2,4-D and Kin with 0.61 g fresh weight callus while, the lowest weight was observed in the same PGRs, but in explants derived from March corms (0.33 g). The most appropriate PGRs for corm callogenesis in March explants were reported with 2,4-D and BA whereas no significant difference was observed in this hormonal treatment in May and March (Fig. 4).

The interaction among four PGRs groups at nine different concentrations on callus induction on corm derived explant was shown in Figure 5. According to the results of this figure, among the tested PGRs groups, two combination including 4 mgL^{-1} 2,4-D + 1 mgL^{-1} Kin and 8 mgL^{-1} NAA + 1 mgL^{-1} Kin were significantly effective on saffron corm callogenesis with 1.07g and 1.01g callus fresh weight, respectively (Fig. 5).

The interaction of different corm developmental stages, Medium types and PGRs groups on saffron corm callogenesis was shown in table 3. The results showed that there is a statistically significant interaction among the factors mentioned and also present the pioneer combination of all factors for the purpose of saffron callogenesis in which the explants derived from corms harvested in May cultured in B_5 basal medium supplemented with 4 mgL^{-1} 2,4-D + 1 mgL^{-1} Kin or 8 mgL^{-1} NAA+ 1 mgL^{-1} Kin resulted in the higher fresh weight of callus for establishing saffron cell suspension cultures (Table 3).

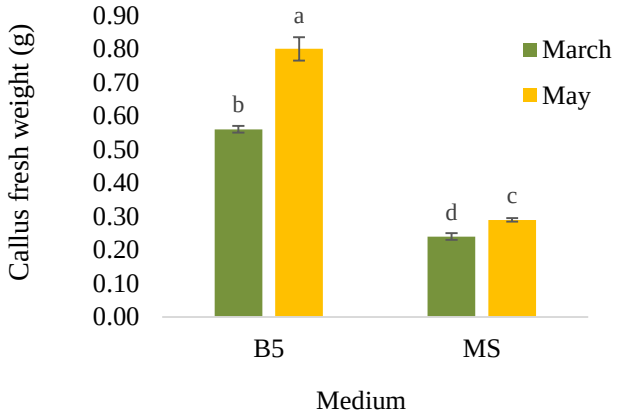

Fig. 3. The interaction effect of saffron corm developmental stage (harvested in March and May) and types of medium on callus fresh weight of saffron after two months. Different letters indicate significant differences between treatments ($p \leq 0.05$).

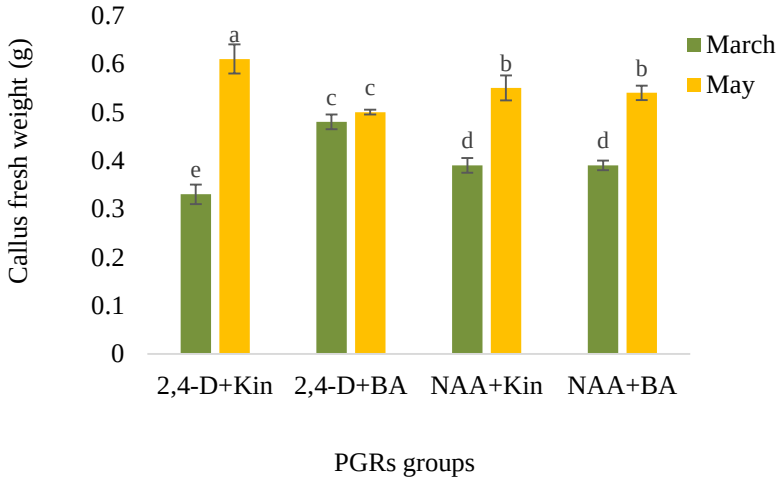

Fig. 4. The interaction of saffron corm developmental stages (March and May harvesting) and PGRs groups types on callus fresh weight of saffron after two months. Different letters indicate significant differences between treatments ($p \leq 0.05$).

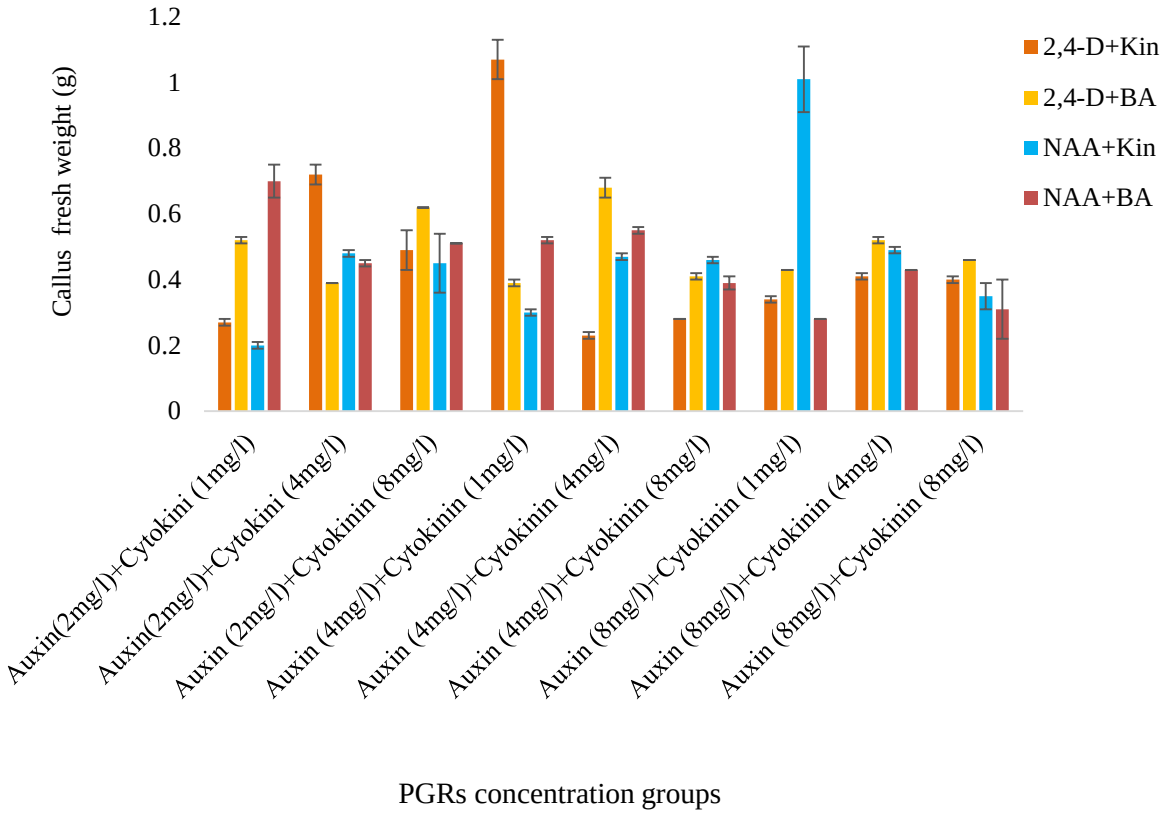

Fig. 5. The interaction effect of different PGRs groups and their concentrations on callus fresh weight of saffron after two months ($p \leq 0.05$). Error bars indicates standard deviation of the mean

Table 3. The interaction effect of three factor including different corm developmental stage, culture media types and PGRs groups on callus fresh weight of saffron after two months.

		Immature explant corm (harvested in March)		Mature explant corm (harvested in May)	
		B_5	MS	B_5	MS
PGRs groups	2,4-D+Kin	0.43 ^{e}	0.23 ^{gh}	0.93 ^{a}	0.28 ^{fg}
	2,4-D+BA	0.69 ^{c}	0.28 ^{fgh}	0.68 ^{c}	0.32 ^{f}
	NAA+Kin	0.55 ^{d}	0.22 ^{h}	0.82 ^{b}	0.28 ^{fg}
	NAA+BA	0.55 ^{d}	0.22 ^{h}	0.78 ^{b}	0.29 ^{f}

DISCUSSION

Generally, the induction and growth of callus in plant tissue culture depends on the types and concentration of plant growth regulators used in the medium. In addition to that, the ratio of the auxin to cytokinin also plays an important role in callogenesis (Amini et al., 2013). Accordingly, the type and the best levels of these PGRs as well as their interaction to the other factors such as type and age of explants should be investigated. Till now, several researches have studied the effect of different types and levels of PGRs on saffron callus induction (Dahr & Sapru 1993; Karaoğlu et al., 2006; Sharma et al., 2008; Blazquez et al., 2009; Georgiev et al., 2009). These studies show that the impacts of a variety of basal medium supplemented with different PGRs combination have been investigated on saffron callogenesis in which some outputs confirm the results of this study and in some cases they show a deviation. In general,

the results of previous researches showed that the MS culture medium supplemented with PGRs combination like 1 mgL^{-1} 2,4-D + 2 mgL^{-1} BAP (Safarnejad et al., 2016); 2,4-D with BAP both at 0.5 mgL^{-1} plus 2 % coconut milk (Ilahi et al., 1987); MS medium supplemented with 4 mgL^{-1} NAA + 4 mgL^{-1} TDZ (Verma et al., 2016), combination of TDZ (0.5, 1 mgL^{-1}) and NAA (2, 3 mgL^{-1}) (Moradi et al., 2018) and MS medium supplemented by BAP (1 mgL^{-1}) plus 2,4-D (1 mgL^{-1}) (Halim et al., 2018) were all suitable PGRs combinations for callus induction in saffron corm segments. Vahedi et al. (2014) also reported that a combination of 2 mgL^{-1} 2,4-D + 1 mgL^{-1} BA followed by 1 mgL^{-1} 2, 4-D + 0.15 mgL^{-1} Kin showed the best treatment for callus induction on corm derived explants. Obviously, in most of these reports, the use of 2,4-D as an auxin was dominant for saffron corm callogenesis. These results confirm the findings of the present study in which, combination of 2,4-D and Kin were introduced as the appropriate auxin for corm callogenesis in saffron.

Although, in several studies, MS has been selected as the main basal medium for *in vitro* culture of saffron (Ilahi et al., 1987; Safarnejad et al., 2016; Verma et al., 2016; Halim et al., 2018; Moradi et al., 2018), there are few reports on comparison of different media on corm callogenesis of *C. sativus* (Chen et al., 2003) or even in other *Crocus* species (Verma et al., 2016). Chen et al. (2003) in addition to examining the various types of PGRs, they checked out the basal medium types in saffron cell cultures. Their results showed that among the investigated five media, B_5 medium produced the maximum cell biomass. In the present study, the comparison of B_5 and MS, not only statistically showed the priority of B_5 on biomass, but also its influence on production of more colorants was apparent (data not shown). Most of the researchers have emphasized on the role of PGRs level and basal medium on saffron corm callogenesis while in addition to medium and PGRs conditions, the type and kind of explant is also very influential on saffron callogenesis (Sajjadi & Pazhouhandeh 2015). An experiment was carried out to investigate the callus induction in mother and daughter saffron corms in liquid and semi-solid medium. Their results showed that the average time of callus induction in daughter corms explants was lower in comparison with the mother corms and this relation was vice versa in case of frequency of induction (Suarez-Ambriz et al., 2009). Although in that research it was not mentioned that the collected daughter corm was at which developmental stage, as they were not fully developed or mature, it can be stated that their results confirmed the present study in case of callus induction starting and percentage.

As already mentioned, researchers believed that callus induction and proliferation depend on the type and developmental stage of the explant (Holme & Petersen, 1996). Although the results of the present study confirmed that the physiological age has a complementary role in selecting the best medium and for a successful callogenesis, but in the role of explant developmental stage on callogenesis, the reported results are varied. Some scientific findings revealed that the explant developmental stage can effect on callus induction through the small RNA-mediated regulation. Accordingly, it was concluded that immature explant has more potential ability to callogenesis compare to mature explant (González et al. 2019). In confirmation to them, some other researchers stated that the younger explants were more efficient in callus induction (Hoque & Mansfield 2004). But in contrast to the previous two studies, the results of another study showed that intermediate explant age can create better conditions for callus proliferation than young explant (Aisagbonhi et al., 2015). Apparently, some of these results do not support our findings, but it is clear that the used explants in this study are completely different from those cultured in their experiments. In botanically point of view, corm is a storage organ. It means that a corm at the mature stage or fully grown (fully developed) should contain more active cells due to having higher levels of indigenous hormones. In this study, the mature saffron corms have been harvested in May which is at fully development and growing stage then followed by dormancy and initiation of bud flowers

afterward. It means corms should be at high contents of nutrition and indigenous hormones to support the coming organs. Accordingly it is expectable to observe better results in callus induction of mature corm explants than immature ones.

CONCLUSION

According to the obtained results in this study it can be concluded that an efficient callus induction protocol is a prerequisite for *C. sativus* tissue and cell culture. This study for the first attempt, tried to evaluate the effect of different saffron corm developmental stages on callus induction. In the present study, it was found that May is the promising time for preparing saffron corms as source of explant material for callogenesis. It appeared that efficient saffron cell biomass production is related to the mature stage of corms. In this research, significant differences in callogenesis capacity of different media were also observed. In general, B_5 medium in comparison with MS medium illustrated more suitability for callus induction in saffron corms. In addition to the maturity of corms and medium type, it was found that among the several plant growth regulators that were tested for callus induction, 2,4-D (4 mgL^{-1}) and Kin (1 mgL^{-1}) combination was the optimum formulation.

As shown in figures in this study, it is obviously observed that the induced cells are colored, which demonstrates the presence of pigments. However, further investigations are needed to identify secondary metabolites in produced callus. Also, the production of saffron secondary metabolites through cell suspension culture can be considered in subsequent experiments. The results also revealed that there are still two problematic issues due to the presence of compact callus in some treatments as well as achievement of organogenesis or embryogenesis in some other treatments which need to be concerned. These phenomena are mostly observed in treatments containing NAA as auxin PGRs. Therefore, suggested that the association of plant growth regulators and their levels in culture medium with nature of produced callus (friable or compact cells, organogenesis and embryogenesis calli and etc.) should be investigated in the future studies.

Acknowledgements
The authors would like to thank the Department of Food Biotechnology at the Research Institute of Food Science and Technology (RIFST), Mashhad, Iran, for laboratory facilities, Post-Graduate Research Fellowship and financial support.

Conflict of interest
The authors declare that there is no conflict of interest.

REFERENCES

Abbas, M. S., El-shabrawi, H. M., Soliman, A. S. & Selim, M. A. (2018) Optimization of germination, callus induction, and cell suspension culture of African locust beans *Parkia biglobosa* (Jacq.) Benth. *Journal of Genetic Engineering and Biotechnology*, *16*(1), 191-201. https://doi.org/10.1016/j.jgeb.2017.10.012

Aisagbonhi, E. P., 1Isalar, C. E., 1Odenore, V. D., 1Ogbebor, J. U., 1Eke, C. R., Asemota, O. and Shittu, H. O. (2015). The interplay between explant developmental stages and phytohormone type in callogenesis of shea tree (*Vitellaria paradoxa* C. F. Gaertn). *European International Journal of Science and Technology*, *4*(7), 50-57. https://www.researchgate.net/publication/303082490

Amini, F., Ghanbarzadeh, Z. & Askary Mehrabadi, M. (2013). Optimization of callus production and plant regeneration in *Salsola arbuscular* pall. *Journal of Cell and Tissue*, *4*(2), 129-137.

Antonelli, M. & Druart, P. (1990). The use of a brief 2, 4-D treatment to induce leaf regeneration on *Prunus canescens*. *Acta Horticulturae*, *280*, 45–50. https://doi.10.17660/ActaHortic.1990.280.6

Blazquez, S., Olmos, E., Hernandez, J. A., Fernandez-Garcia, N., Fernandez, J. A. & Piqueras, A. (2009). Somatic embryogenesis in saffron (*Crocus sativus* L.) Histological differentiation ad implication of some components of the antioxidand enzymatic system. *Plant Cell Tissue Organ Culture*, *97*(1), 49-57. https://doi.10.1007/s11240-009-9497-y.

Chen, S., Wang, X., Zhao, B., Yuan, X. & Wang, Y. (2003). Production of crocin using *Crocus sativus* callus by two-stage culture system. *Biotechnology Letters* 25(15):1235-1238

Choob, V., Vlassova, T., Butenko, R. (1994). Callusogenesis and morphogenesis in generative organ culture of the single flowering species of crocus. *Russian Journal of Plant Physiology*, *41*(6), 712-716.

Dayal, S., Lavanya, M., Devi, P. & Sharma, K. K. (2003). An efficient protocol for shoot regeneration and genetic transformation of Pigeon pea [*Cajanus cajan* (L.) Millsp.] using leaf explants. *Plant Cell Reports*, *21*, 1072–1079. https://doi.org/10.1007/s00299-003-0620-y

Devi, K., Sharma, M. & Ahuja, P. (2014).Direct somatic embryogenesis with high frequency plantlet regeneration and successive cormlet production in saffron (*Crocus sativus* L.). *South African Journal of Botany*, 93, 207-216. https://doi.org/10.1016/j.sajb.2014.04.006

Dhar, A. & Sapru, R. (1993). Studies on saffron in Kashmir. In vitro production of corm and shoot like structures. *Indian Journal of Genetics and Plant Breeding*, 53(2), 193-196.

Fallahi, H.R., Mahmoodi, S. (2018). Impact of water availability and fertilization management on saffron (*Crocus sativus* L.) biomass allocation. *Journal of Horticulture and Postharvest Research*, *1*(2), 131-146.

Gamborg, O. L., Miller, R. A., Ojima, K. (1968). Nutrient requirements of suspension cultures of soybean root cells. *Experimental Cell Research*, *50*(1), 151-158. https://doi.org/10.1016/0014-4827(68)90403-5

Georgiev, M. I., Weber, J. & Maciuk, A. (2009). Bioprocessing of plant cell cultures for mass production of targeted compounds. *Applied Microbiology and Biotechnology*, *83*(5), 809-823. https://doi.org/10.1007/s00253-009-2049-x

Halim, R., Akyol, B., Gurel, A. & Bayraktar, M. (2018). In vitro callus induction of saffron (*Crocus sativus* L.). *International Journal of Innovative Research in Science, Engineering and Technology*, 3(11), 1-5.

Holme, I. B., Petersen, K. K. (1996). Callus induction and plant regeneration from different explant types of *Miscanthus x ogiformis* Honda 'Giganteus'. *Plant Cell Tissue and Organ Culture*, *45*(1), 43-52. https://doi.org/10.1007/bf00043427

Hoque, E., Mansfield, J. W. (2004). Effect of genotype and explant age on callus induction and subsequent plant regeneration from root-derived callus of Indica rice genotypes. *Plant Cell, Tissue and Organ Culture*, 78, 217-223.

Husaini, A. M., Kamili, A. N., Wani, M., Teixeira da Silva, J. & Bhat, G. (2010). Sustainable saffron (*Crocus sativus* Kashmirianus) production. Technology and policy interventions for Kashmir. *Functional Plant Science and Biotechnology*, *4*(2), 116-127.

Ibrahim, R. & Debergh, P.C. (2001). Factors controlling high efficiency adventitious bud formation and plant regeneration from in vitro leaf explants of roses (*Rosa hybrida* L.). *Scientia Horticulturae*, *88*, 41–57. https://doi.org/10.1016/s0304-4238(00)00189-8

Ilahi, I., Jabeen, M. & Firdous, N. (1987). Morphogenesis with saffron tissue culture. *Journal of Plant Physiology*, *128*(3), 227-232. https://doi.org/10.1016/s0176-1617(87)80236-5

González, G. A., Pacheco, M. G.Cecilia, Oneto, D., Etchart, V. J., Kandus, M. V., Salerno, J. C., Eyherabide, G., Presello, D., Lewi, D. M. (2012). Somatic embryogenesis and plant regeneration capacity in *Argentinean maize* (*Zea mays* L.) inbred lines. *Electronic Journal of Biotechnology*, *15*, 1-15.

González, J, V. T., López-Ruiz, B. A., Baldrich, P., Luján-Soto, E., Meyers, B. C., Dinkova, T. D. (2019). The explant developmental stage profoundly impacts small RNA-mediated regulation at the

dedifferentiation step of maize somatic embryogenesis. *Scientific Reports*, 9 (14511), 1-14. https://doi.org/10.1038/s41598-019-50962-y

Karaoğlu, C., Çocu, S., Ipek, A., Parmaksız, I., Uranbey, S., Sarıhan, E., Arslan, N., Kaya, M. D., Sancak, C., Ozcan, S., Gurbuz, B., Mirici, S., Er, C. & Khawar, K. M. (2006). In vitro micropropagation of saffron. *In II International Symposium on Saffron Biology and Technology*, *739*, 223-227. https://doi.org/10.17660/actahortic.2007.739.28

Lopez-Corcoles, H., Brasa-Ramos, A., Montero-García, F., Romero-Valverde, M. & Montero-Riquelme, F. (2015). Phenological growth stages of saffron plant (*Crocus sativus* L.) according to the BBCH Scale. *Spanish Journal of Agricultural Research, 13*(3), 1-7. https://doi.org/10.5424/sjar/2015133-7340

Lozano, P., Delgado, D., Gomez, D., Rubio, M., Iborra, J. (2000). A non-destructive method to determine the safranal content of saffron (*Crocus sativus* L.) by supercritical carbon dioxide extraction combined with high performance liquid chromatography and gas chromatography. *Journal of Biochemical* and *Biophysical Methods*, *43*(1-3), 367-378. https://doi.org/10.1016/s0165-022x(00)00090-7

Mathew, B. (1977). *Crocus sativus* and its allies (Iridaceae). *Plant Systematic and Evolution*, *128*(1-2), 89-103. https://doi.org/10.1007/bf00985174

Mazumdar, P., Basu, A., Paul, A., Mahanta, C. & Sahoo, L. (2010). Age and orientation of the cotyledonary leaf explants determine the efficiency of de novo plant regeneration and Agrobacterium tumefaciens mediated transformation in *Jatropha curcas* L. *South African Journal of Botany*, 76, 337–344. https://doi.org/10.1016/j.sajb.2010.01.001

Milyaeva, E., Azizbekova, N. S., Komarova, E. & Akhundova, D. (1995). *In-vitro* formation of regenerant corms of saffron (*Crocus sativus* L.). *Russian Joural of Plant Physiology*, *42*(1), 112-119.

Mir, J. I., Abuzar, A., Wani, S. H., Ahmad Sheikh, M., Rashid, R. & Mir, H. (2010). In vitro development of microcorms and stigma like structures in saffron (*Crocus sativus* L.) *Physiology and Molecular Biology of Plants*, *16*(4), 369-73. https://doi.org/10.1007/s12298-010-0044-4

Moradi, A., Zarinkamar, F., Caretto, S. & Azadi, P. (2018). Influence of thidiazuron on callus induction and crocin production in corm and style explant of *Crocus sativus* L. *Acta Physiology Plant*, *40*(11), 185. https://doi.org/10.1007/s11738-018-2760-2

Murashige, T. & Skoog, F. (1962). A revised medium for rapid growth and bio assays with tobacco cultures. *Plant Physiology*, *15*, 473-497. https://doi.org/10.1111/j.1399-3054.1962.tb08052.x

Murthy, H. N. & Pyati, A. N. (2001). Micropropagation of *Aerides maculosum* lindl. (Orchidaceae). *In Vitro Cellular and Developmental Biology-Plant*, *37*, 223-226. https://doi.org/10.1007/s11627-001-0039-5

Mzabri, I. Addi, M. & Berrichi, A. (2019). *Traditional and Modern Uses of Saffron (Crocus sativus). Cosmetics*, *6*(63), 1-11. https:doi: //10.3390/cosmetics6040063

Plessner, O., Negbi, M., Ziv, M. & Basker, D. (1989). Effect of temperature on the flowering of the saffron crocus (*Crocus sativus* L.): induction of hysteranthy. *Israel Journal of Plant Sciences*, *38*(1), 1-7. https://doi.org/10.1007/978-3-642-73271-3_91

Raja, W., Zaffer, G. & Wani, S. (2006). In vitro micro corm formation in saffron (*Crocus sativus* L.) In: *II International Symposium on Saffron Biology and Technology*, *739*, 291-296. https://doi.org/10.17660/actahortic.2007.739.37

Safarnejad, A., Alamdari, S. B. L., Darroudi, H. & Dalir, M. (2016). The effect of different hormones on callus induction, regeneration and multiplication of saffron (*Crocus sativus* L.) corms. *Saffron Agronomy & Technology*, *4*(2), 143-154.

Sajjadi, M. & Pazhouhandeh, M. (2015). Study on effect of type of explant and hormone on callus induction and regeneration in saffron (*Crocus sativus* L.). *Saffron Agronomy and Technology*, *3*(3), 195-202.

Sharifi, G., Ebrahimzadeh, H., Ghareyazie, B. & Karimi, M. (2010). Globular embryo-like structures and highly efficient thidiazuron-induced multiple shoot formation in saffron (*Crocus sativus* L.*). In Vitro Cellular and Developmental Biology – Plant*, *46*(3), 274-280. https://doi.org/10.1007/s11627-009-9264-0

Sharma, K., Rathour, R., Sharma, R., Goel, S., Sharma, T. & Singh, B. (2008). In vitro cormlet development in *Crocus sativus*. *Plant Biology*, *52*(4), 709-712. https://doi.org/10.1007/s10535-008-0136-y

Suarez-Ambriz, I., Gonzalez-Ronquillo, M., Dominguez-Lopez, A., Trejo-Gonzalez, A. & Riveron-Negrete, L. (2009). A method to improve the induction of callus from saffron (*Crocus sativus* Linneo) corm. *In: II International Symposium on Saffron: Forthcoming challenges in cultivation research and economics*, 850, 99-102. https://doi.org/10.17660/actahortic.2010.850.14

Sun, Y., Zhao, Y., Wang, X., Qiao, G., Chen, G., Yang, Y., Zhou, J., Jin, L. & Zhuo, R. (2009). Adventitious bud regeneration from leaf explants of *Platanus occidentalis* L. and genetic stability assessment. *Acta Physiologia Plantarum*, *31*, 33–41. https://doi.org/10.1007/s11738-008-0196-9

Thomas, T. D. (2003). Thidiazuron induced multiple shoot induction and plant regeneration from cotyledonary explants of mulberry. *Biologia Plantarum*, *46*, 529–533. https://doi.org/10.1023/a:1024807426591

Ting, P., Pai, S., Wu, I. & Wang, P. (1979). Preliminary report on tissue culture of corm of *Crocus sativus*. *Acta Botanica Sinica*, *21*, 387-387.

Vahedi, M., Kalantari, S. & Salami, S.A. (2014). Factors affecting callus induction and organogenesis in saffron (*Crocus sativus* L.). *Plant Tissue Culture and Biotechnology*, *24*(10), 1-9. https://doi.org/10.3329/ptcb.v24i1.19184

Veraplakorn, V. (2016). Micropropagation and callus induction of *Lanata camara* L.: a medicinal plant. *Agriculture and Natural Resources*, *50*(5), 338-344. https://doi.org/10.1016/j.anres.2016.12.002

Verma, S. K., Das, A. K., Cingoz, G. S., Uslu, E., Gurel, E. (2016). Influence of nutrient media on callus induction, somatic embryogenesis and plant regeneration in selected Turkish crocus species. *Biotechnology Report*, *10*, 66-74. https://doi.org/10.1016/j.btre.2016.03.006

Xu, L., Najeeb, U., Raziuddin, R., Shen, W. Q., Shou, J. Y., Tang, G. X. & Zhou, W. J. (2009). Development of an efficient tissue culture protocol for callus formation and plat regeneration of wetland species *Juncus effuses* L. *In Vitro Cellular and Developmetal Biology Plant*, *45*(5), 610-618. https://doi.org/10.1007/s11627-009-9228-4

Yildrim, E. (2007). Development of *in vitro* micropropagation techniques for saffron (*Crocus sativus* L.). MSc. Thesis, Middle East Technical University, Ankara, Turkey.