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Improvement of friable callus induction of *Crocus sativus* L. and establishment of a cell suspension culture system with high biomass

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ABSTRACT

Purpose: This study aims to explore the potential of in vitro culture as a method for scaling up the production of saffron based medicinal compounds, the most expensive spice renowned. Emphasis is placed on the critical role of friable callus (FC) formation as a prerequisite for successful suspension culture. **Research method:** The research primarily investigates FC formation, focusing on the impact of varying strengths of Murashige and Skoog (MS) medium as well as combinations of NAA or 2,4-D and BA or Kin on compact callus. Subsequently, the study involves supplementing the MS medium with different concentrations of 2,4-D, kin, zeatin, glutamine, sucrose, and nitrogen to establish a cell suspension culture. **Findings:** The highest FC yield was achieved on a solid medium containing 2,4-D (1 mg l^{-1})+Kin (0.2 mg l^{-1}), resulting in a fresh weight (FW) of 0.413 g. Furthermore, MS combined with 2,4-D (1 mg l^{-1})+Kin (0.2 mg l^{-1})+glutamine (10 mg l^{-1}), as well as MS+2,4-D (0.5 mg l^{-1})+zeatin (0.3 mg l^{-1})+glutamine (10 mg l^{-1}), demonstrated the highest FW under suspension conditions. The study also identified that 30 g l^{-1} sucrose and 30 µM were optimal for inducing maximum FW. **Research limitations:** Cell biomass is influenced by several factors that should to be optimized. **Originality/Value:** This research concludes that a cell suspension system holds promise for rapidly generating sufficient cell biomass to produce valuable secondary metabolites within a limited timeframe and space. Notably, the system successfully increased biomass from 0.2 to 1.2 g, underscoring its potential for efficient saffron-based product development.

INTRODUCTION

In contemporary research, there is a growing interest in identifying alternative methods that can effectively reduce costs and enhance the efficiency of producing secondary metabolites. One such successful method is the use of plant Cell Suspension Culture (CSC), as highlighted by Pant (2014). CSC has demonstrated the capability to generate natural compounds at the rates comparable to, or even higher than the intact plants, presenting a significant advantage in terms of quantity (Ziaratnia et al., 2009). A noteworthy benefit of CSC is its ability to ensure the continuous production of valuable compounds, unaffected by geographical or seasonal constraints, thus distinguishing it from conventional field cultivation practices (Rao & Ravishankar, 2002).

To establish an effective CSC system, the formation of Friable Callus (FC) with homogeneous cells is imperative (Mustafa et al., 2011). Friability, a morphological characteristic of callus, attributes to loosely aggregated cells with lower density (Souza et al., 2014). Successful callogenesis relies on appropriate explant selection, optimization of medium compositions, and determining the type and concentrations of Plant Growth Regulators (PGRs). Among various chemical factors influencing FC production, hormonal combinations and basal medium composition are identified as crucial contributors (Thacker et al., 2018). Additionally, the type of explant is reported to play a significant role in callus initiation (Dar et al., 2021).

Several studies have reported FC production in various plants. For instance, Keng et al. (2010) successfully produced FC from leaf-derived explants of *Artemisia annua* by culturing them on MS medium supplemented with benzyl adenine (BA) (0.5 mg l-1) and naphthalene acetic acid (NAA) (0.5 mg l-1). Furthermore, Dar et al. (2021) demonstrated the significant impact of different PGR concentrations and combinations on callus induction and maintenance from leaf and root explants of *Atropa acuminate*. Notably, leaf explants exhibited a higher production of friable calli, and reducing NAA levels in the medium resulted in significantly higher friable callus production.

In a CSC system, the consideration of secondary metabolite production becomes high when biomass reaches its maximum level. To achieve this, the improvement of callogenesis, FC production, and subsequent optimization of CSC must be undertaken to attain desired levels of biomass and secondary metabolites. Given the plant species-specific responses to CSC conditions, factors such as PGR type and level, organic and inorganic medium compositions (e.g., nitrogen and phosphate), and sucrose concentrations should be systematically assessed (Murthy et al., 2014; Thacker et al., 2018). Notably, scientists have highlighted the influential role of medium constituents, PGRs, and nitrogen sources in optimizing the callus formation and cell culture of various plant species, such as *Dracocephalum polychaetum*, *D. kotschyi*, and *Artemisia. annua* (Taghizadeh et al., 2020; Keng et al., 2010).

Apart to optimizing medium constituents, several reports reveal the efficacy of adding organic materials, including amino acids like glutamine, in stimulating growth across different plant cells, such as *Juniperus excelsa*, *Catharanthus roseous*, *Hyoscyamus muticus*, and *Glycine max* (Shanjani 2003; Scragg et al., 1990; Basu & Chand 1998; Gueven & Knorr 2011).

Despite numerous reports on saffron corm callogenesis and cell culture optimization, particularly in studies by Verma et al. (2016), Moshtaghi (2020), Taghizadeh et al. (2020), Amini et al. (2022), and Ramandi et al. (2022), the production of saffron friable callus a crucial step for successful cell suspension culture remains unexplored. Therefore, this study was designed to investigate the optimization of solid MS medium components, PGR types,

and concentrations for friable callus production. The ultimate goal is to establish a robust CSC system that enhances saffron cell biomass production.

MATERIALS AND METHODS

This study was conducted at the Research Institute of Food Science and Technology (RIFST), Mashhad, Iran, from 2020 to 2022. Since friable calli production is an imperative step to establish a successful cell suspension culture; therefore, in the first step, the possibility of FC production from the corm-derived compact cells was evaluated in the solid medium by changing in PGRs types and concentrations. The second experiment was focused on optimizing CSC conditions to increase cell biomass. The latter experiment investigated the effect of different factors, such as medium constituent strengths, sucrose, PGRs, and glutamine at different levels. All media components were purchased from Sigma (Japan) and Merck (USA) companies.

Plant materials

In current study, mature corms of saffron were used as plant material for callus induction. The disinfection steps of corms were carried out according to the method of Ziaratnia and Amini (2021). The callus was prepared from a preliminary experiment in which different PGRs at several concentrations were used for higher callus induction. Among all treatments, a combination of NAA and BA (NAA, 8 mg l^{-1} and BA, 1 mg l^{-1}) was the best regarding fresh weight and reddish colour (data not published), but the calli were compact. Therefore, the compact calli from this treatment were selected as an explant for further investigations in this study.

FC induction on solid medium

To produce friable calli and desirable biomass, the compact corm derived-callus from solid MS medium was sub-cultured on MS medium with lower levels of PGRs than those in callus induction. Media were supplemented with different combinations of 2,4-dichlorophenoxyacetic acid (2,4-D) or NAA (0.5 and 1 mg l^{-1}) as auxins and Kin or BA (0.2, 0.5 and 1 mg l^{-1}) as cytokinins (Table 1).

Table 1. Plant growth regulators treatments for friable callus formation in solid MS medium.

Treatment	2,4-D (mg l^{-1})	Kin (mg l^{-1})	BA (mg l^{-1})	Treatment	NAA (mg l^{-1})	Kin (mg l^{-1})	BA (mg l^{-1})
E1	0.5	0.2	-	F1	0.5	0.2	-
E2	0.5	0.5	-	F2	0.5	0.5	-
E3	0.5	1	-	F3	0.5	1	-
E4	1	0.2	-	F4	1	0.2	-
E5	1	0.5	-	F5	1	0.5	-
E6	1	1	-	F6	1	1	-
E7	0.5	-	0.2	F7	0.5	-	0.2
E8	0.5	-	0.5	F8	0.5	-	0.5
E9	0.5	-	1	F 9	0.5	-	1
E10	1	-	0.2	F10	1	-	0.2
E11	1	-	0.5	F11	1	-	0.5
E12	1	-	1	F12	1	-	1

The medium pH was adjusted to 5.7, and then solidified by 0.7% agar before autoclaving at 121°C for 15 minutes. Cultures were incubated at 22±2 °C in dark conditions. After four weeks, all treatments were statistically analysed based on cell fresh weight and the percentage of organogenesis or embryogenesis. The friability and yellow to red-coloured intensities of the calli were evaluated visually. The friability was determined as an easily friable callus with no apparent regenerated organ. Treatments with desirable friability with no organogenesis and higher cell biomass were used to establish and optimize CSC in the next experiments.

CSC establishment and adaptation

In this part, four-week-old FC with yellow colour related to the superior treatments from the previous experiment was pooled together and used as initial cells for the establishment of CSC. To make homogeneity and adapt the cells to the new conditions, 0.5 g of the pooled cells were inoculated into 100 ml Erlenmeyer flask containing 20 ml liquid MS medium supplemented with an optimum concentration of 2,4-D and kin (0.5 and 0.2 mg l^{-1} respectively). Cultures were then placed on an orbital shaker at 110 rpm at 20±2 ºC in dark conditions for four weeks.

CSC medium optimization by changing PGRs type and glutamine concentration

This part evaluated the effect of MS medium (Full MS, ½ MS), PGRs type (2,4-D, Kinetin, Zeatin), and glutamine at different levels. Table 2 shows the different treatments prepared in this part of the experiment.

At first, 0.2 g adapted cells were inoculated into the 100 ml flasks containing 20 ml liquid MS in different strengths with a content of 3.0 % sucrose and MS vitamins. Thereafter, flasks were placed on an orbital shaker as mentioned before. For analysis of biomass accumulation, data was recorded as fresh and dry weight (g) every week for nine weeks.

CSC optimization by changing the levels of sucrose and nitrogen

In this part, different levels of sucrose and nitrogen were examined to reach the higher cell biomass (Table 3). MS medium supplemented with 2,4-D (0.5 mg l^{-1}), Zeatin (0.3 mg l^{-1}), and Glutamine (10 mg l^{-1}) selected from the previous experiment were similar for all treatments. Treated factors were evaluated based on the rate of cell growth over nine weeks.

Table 2. Different treatments for cell suspension culture optimization of saffron (*Crocus sativus* L.).

Treatment	MS strength	2,4-D (mg l^{-1})	Kin (mg l^{-1})	Zeatin (mg l^{-1})	Glutamine (mg l^{-1})
A1	Full	1	0.2	-	10
A2	Full	1	0.2	-	-
A3	Half	1	0.2	-	10
A4	Half	0.5	-	0.3	10
A5	Full	0.5	-	0.3	10
A6	Full	0.5	-	0.3	-

*Nitrogen sources were NH_4NO_3 and KNO_3.

Table 3. Different levels of sucrose and nitrogen in saffron (*Crocus sativus* L.) cell suspension culture.

Treatment	Sucrose (g l^{-1}) and Nitrogen (µM)
B1	Sucrose (30) + Nitrogen (30)
B2	Sucrose (30) + Nitrogen (60)
B3	Sucrose (30) + Nitrogen (90)
B4	Sucrose (60) + Nitrogen (30)
B5	Sucrose (60) + Nitrogen (60)
B6	Sucrose (60) + Nitrogen (90)

*Nitrogen sources were NH_4NO_3 and KNO_3.

Statistical analysis

The traits of cell fresh weight and the percentage of organogenesis or embryogenesis (in FC induction on solid medium experiment), cell fresh and dry weight (in CSC medium optimization by changing PGRs type and glutamine concentration experiment) and rate of cell growth in CSC optimization by changing the levels of sucrose and nitrogen experiment, were statistically evaluated using a Completely Randomized Design (CRD) with three replications. Data were analyzed with JMP software version 11. Mean comparisons were made using Duncan's new multiple range test and the significant differences among means were shown at 5%. Graphs were drawn with Excel software.

RESULTS AND DISCUSSION

FC formation on solid medium

Analysis of variance showed that there were significant differences among the PGRs treatments on FC formation. Figure 1 shows the results of the fresh callus weight in MS medium. The highest FC formation (0.413 g) was observed in E4, in which 2,4-D and Kin were at 1 and 0.2 mg l^{-1}, respectively. Based on the results, the maximum concentration of 2,4-D with the minimum amount of Kin is a more effective combination in FC formation. Interestingly, the callus in all treatments was red with no obvious differences (Fig. 2).

The observed percentage of callogenesis and organogenesis (Fig. 3) revealed that E1, E4, F8, and F10 had the highest (100 %), while E7 and F3 had the lowest rate (11.11 %). The maximum frequency of organogenesis (100%) was observed in F5 with NAA (1.0 mg l^{-1}) and Kin (0.5 mg l^{-1}) (Fig. 3 and 4).

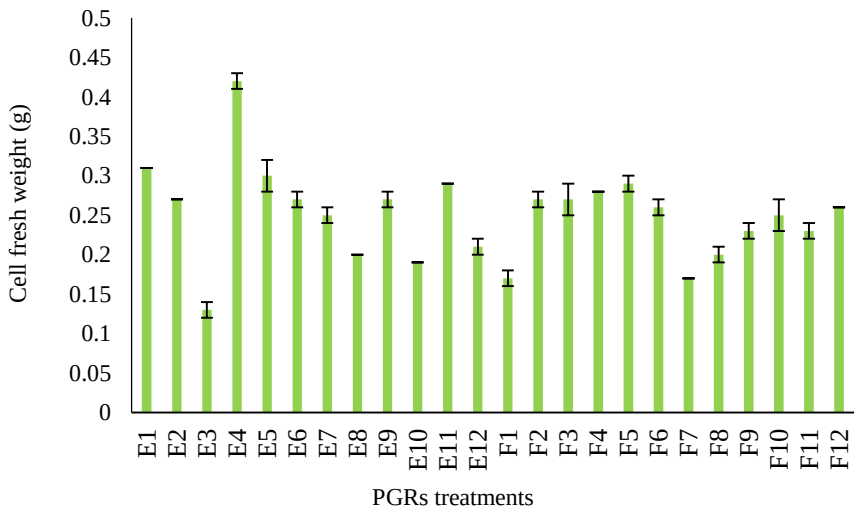

Fig. 1. Effect of different PGRs treatments in solid MS medium on friable callus fresh weight of saffron (*Crocus sativus* L.) ($p \leq 0.05$).

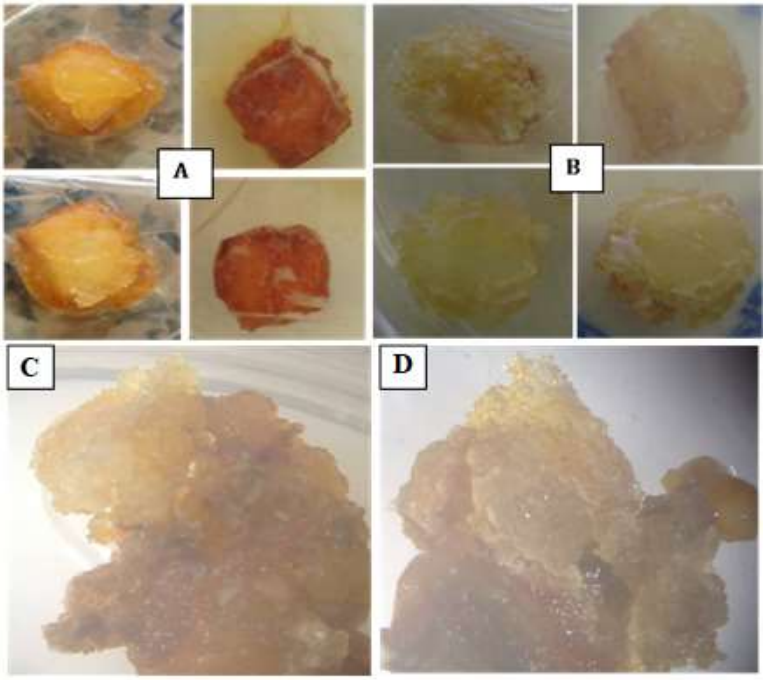

Fig. 2. Compact and friable cells derived from saffron (*Crocus sativus* L.) corm. A) Compact callus (after 2 weeks), B) Compact callus (after 4 weeks), C) Friable cells, Light-red to yellowish, and D) Friable cells, Light-red to whitish.

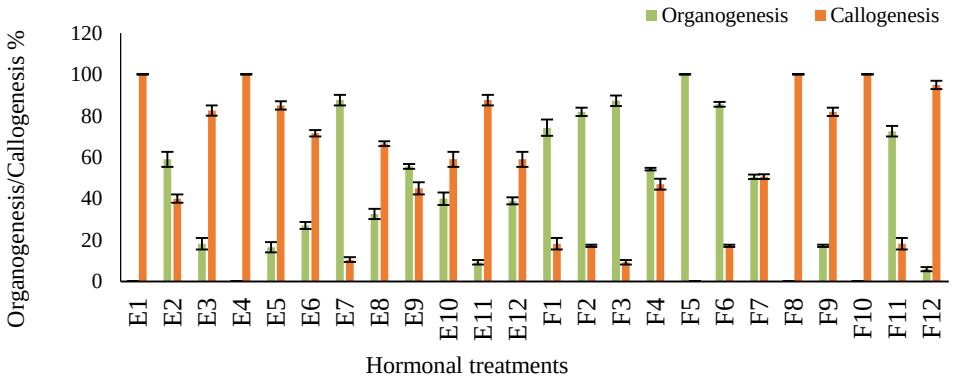

Fig. 3. Effect of different PGRs treatments on saffron corms (*Crocus sativus* L.) organogenesis and callogenesis.

As mentioned, there is no report on FC formation on *C. sativus* corms derived-explants. thus, the results presented here are compared with the findings from other plant species. Several researchers reported different optimized formulas for the formation of fine and small cell aggregates, including NAA and BA (0.25 mg l^{-1}) (Keng et al., 2010), 2,4-D and kin (1 and 1.5 mg l^{-1}) (Osman et al., 2016), or 2,4-D and BA (2 and 0.5 mg l^{-1}) (dos Santos et al., 2017). Although in most of the reports, the type of cytokinin used in FC formation was different, 2,4-D was the dominant type of auxin. It was also revealed that, 2,4-D at lower concentrations than 2 mg l^{-1} is the best concentration for induction of loose cells. It also supports this study's results that 2,4-D at 1 mg l^{-1} was found to be the best for FC formation. Although the results of this study confirmed the efficiency of PGRs on FC formation of saffron, in some cases, these are different, particularly in the types and concentrations of PGRs, which can be attributed to the use of different plant species and organs.

Fig. 4. Organogenesis on corm-derived callus of saffron (*Crocus sativus* L.).

Optimization of medium and CSC establishment

The results of CSC optimization revealed that the medium, PGRs, and glutamine levels could significantly influence cell fresh and dry weight averages. Generally, the sigmoid growth curve was observed in all treatments (Fig. 5). Cells grew vigorously in A1 and A5 treatments. These two media produced the maximum cell fresh and dry weight over nine weeks. All treatments were in a lag phase over the first week. Treatments A1 and A5 started the exponential phase from the second week, while other treatments remained in lag phase until the end of the third week. Cell growth in A1 and A5 in the week of 4 to 6 went to the linear phase. At this stage, the fresh cells' weight raised from 0.42 to 0.77 g in A1 and from 0.42 to 0.75 g in A5. Grown cells in these two treatments reached their maximum weight (0.8 g) in the seventh week. In the week of 7 and 8, their weight remained stable (stationary phase) and finally, in the ninth week, cells entered the death phase, and a decrease in fresh (0.66 g) and dry (0.062 g) weight was observed. Accordingly, the end of the linear phase was in the sixth week (Fig. 5). Therefore, the end of the sixth week is the best time to subculture cells. In the establishment of CSC, A1 and A5, containing the same amount of glutamine, showed a statistically similar effect, while there were different in types and levels of hormones. Although A2 and A6 had similar hormonal combinations to A1 and A5, without glutamine, their fresh and dry weights were significantly lower than A1 and A5 during the tested period. It suggested that hormones alone cannot play a decisive role in cell biomass increment. It means glutamine could be suitable organic nitrogen for saffron cell growth in a CSC system.

Based on the obtained results in this study, A5 containing 2,4-D (0.5 mg l^{-1}), Zeatin (0.3 mg l^{-1}) and glutamine (10 mg l^{-1}) was chosen as a superior treatment for the next experiment as it showed maximum biomass growth. In the second part of the CSC optimization experiment, which was studying different levels of sucrose and nitrogen, it was found that B1 containing sucrose (30 g l^{-1}) and Nitrogen (30 µM) was the best for saffron cell growth (Fig 6). Although in the first week, the highest growth was observed in B5 (0.35 g) and B4 (0.34 g), during the week of 3 to 4, the maximum fresh and dry weight was found to be B1 (1.08 g) and B4 (0.95 g). In the sixth week, B4 entered to the diminishing growth phase, while B1 was remained in the linear phase. The later treatment reached to the maximum fresh weight (1.2 g) in the week of 7. The growth curves of the cells in B1, B2, and B4 show a sigmoid pattern that follow lag, exponential, linear, deceleration, stationery and decline phases. Indeed, the end of exponential phase, similar to the previous experiment, was at the end of sixth week (Fig 6), which is the appropriate time to subculture of the cells or elicitation. In this part, as the same as FC experiment, cells were in maximum biomass level at the week of 7 to 9 and entered to the death stage afterward. This identical sigmoid growth was not visible in other treatments (B3, B5, B6), as their cells growth were very limited.

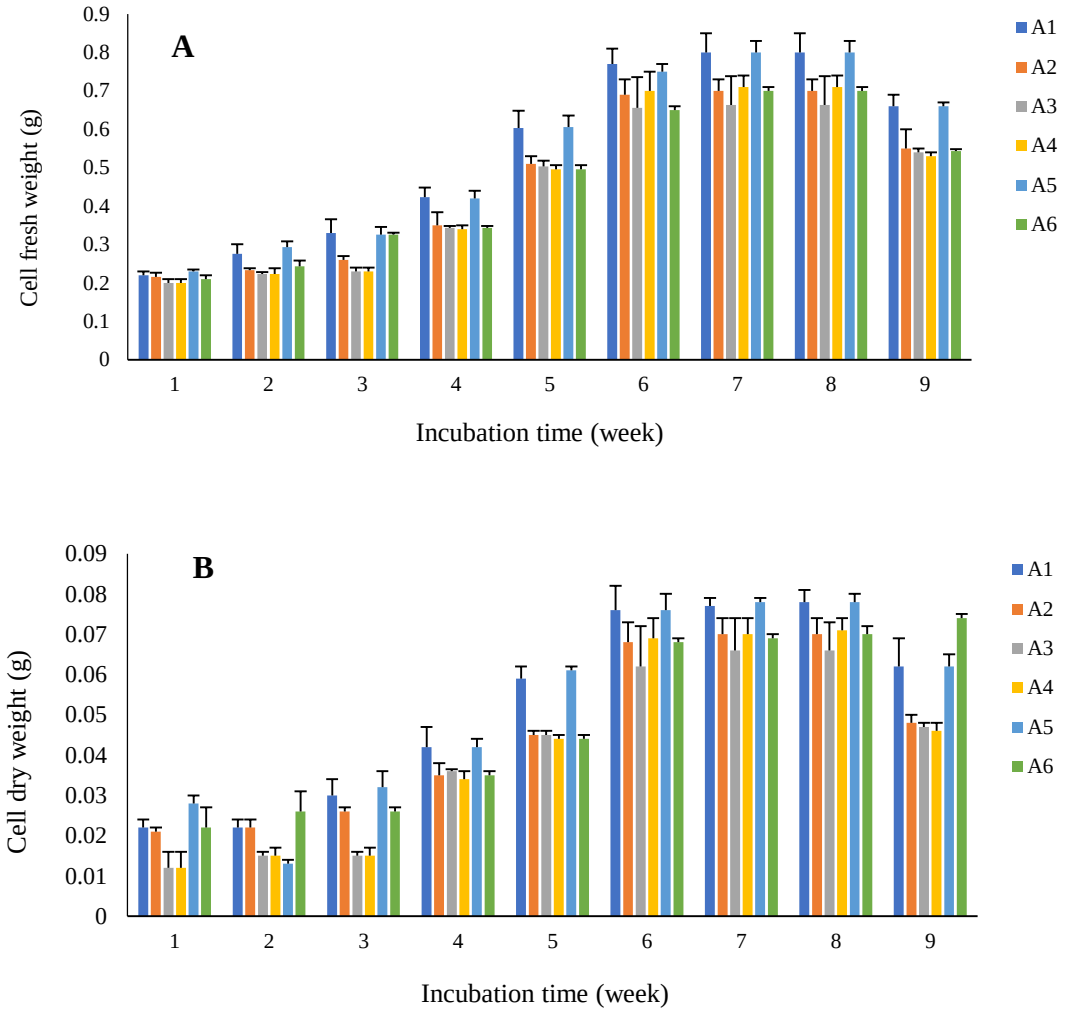

Fig. 5. Effect of different treatments (A1, A2, A3, A4, A5, A6) on weight of saffron (*Crocus sativus* L.) cells in suspension culture system over 9 weeks. A) Cell fresh weight, B) Cell dry weight.

Scientists suggested that PGRs composition, mineral salts, and carbon sources can influence plant cell growth in suspension cultures (Knobloch & Berlin, 1980). Their results revealed that the optimal mineral salts in the medium and hormonal concentration/ratio for cell growth in various cultivars of *Cannabis sativa* are different. Indeed, the type of plant species and the type of cultivar play a decisive role in the selection of medium components (Thacker et al., 2018). The same results have also been reported by Jamil et al. (2018) in which, the establishment of CSC of mangosteen (*Garcinia mangostana*) was found to be faster in the lower concentration of growth regulators than those applied in callus induction. They used MS medium supplemented with 2,4-D and BAP at 1 mg l^{-1} for FC induction while it was 0.5 mg l^{-1} for the establishment of CSC. PGRs concentration reduction to achieve maximum biomass growth during the transfer of cells from solid to liquid medium was also clearly observed in the present study. Notably, in all the research mentioned above, the basal medium used for different plants was MS, which can be concluded that MS is desirable with its macro and micro elements for FC formation. These findings confirm the result of this study in which MS was found to be more suitable than ½ MS.

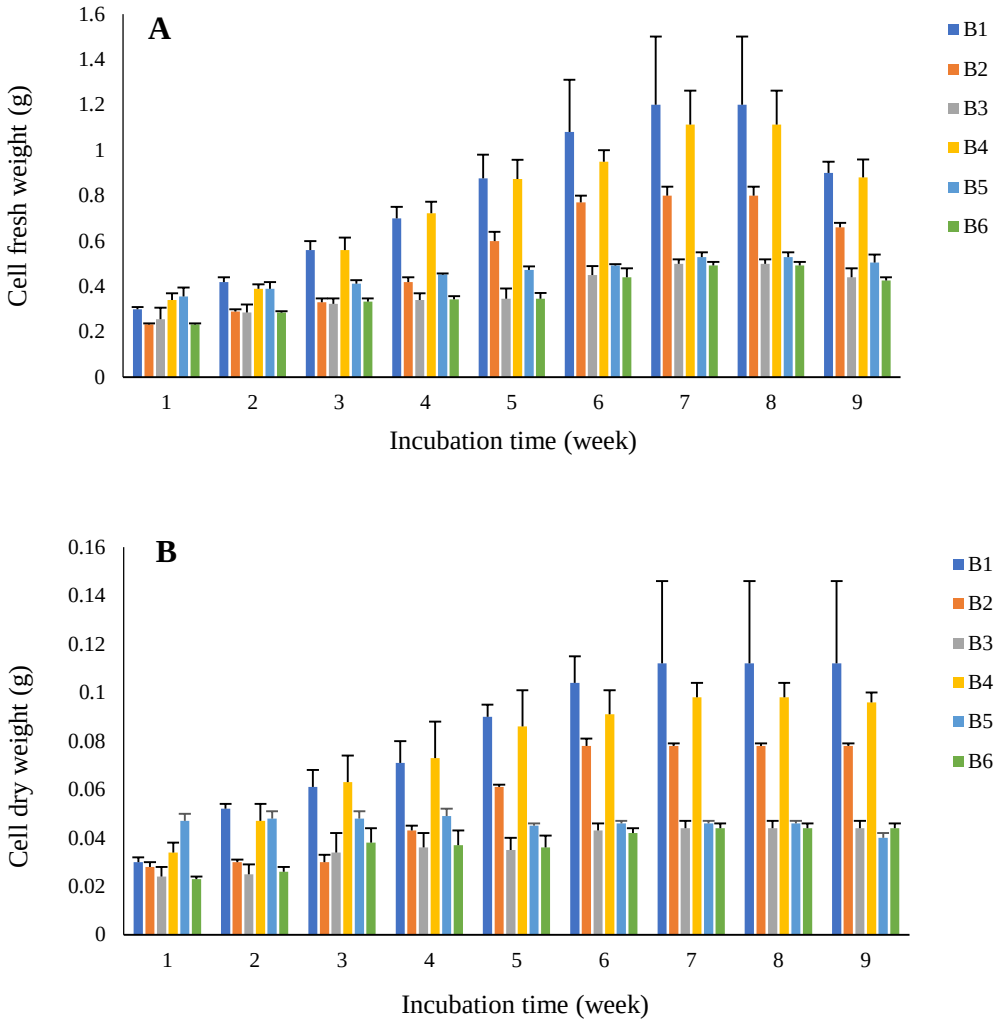

Fig. 6. Effect of different concentrations of sucrose and nitrogen in different treatments (B1, B2, B3, B4, B5, B6) on weight of saffron (*Crocus sativus* L.) cells in suspension culture system over 9 weeks. A) Cell fresh weight, B) Cell dry weight.

Indeed, glutamine and glutamate are the major sources of endogenous amino acids which supply nitrogen for the biosynthesis of nitrogenous compounds such as nucleic acid and proteins in plant cells (Hamasaki et al., 2005). Although the mechanism of glutamine influence on *in vitro* cultures is poorly understood, it is extensively used as a complimentary or sole source of organic nitrogen in plant tissue culture (Marques et al., 2017). This compound is a reduced source of nitrogen that is energetically less costly to assimilate than nitrate or ammonium (Leustek& Kirby 1988). Leustek and Kirby (1988) results clearly indicated that the application of glutamine in protoplast culture acts as a stimulant for cell growth. Hamasaki et al. (2005) found that glutamine, through stimulating the endogenous indole acetic acid (IAA) and isopentenyl adenine (iP), can evoke competence for *in vitro* organogenesis in explants. This study also found that glutamine could increase cell biomass caused by division stimulation.

CONCLUSION

Several factors can influence on increasing efficiency of CSC in order to have higher biomass. Apparently, FC formation is essential to establishing a homogenous CSC system. The results of this study demonstrated that saffron, in addition to the FC formation, as an initial plant material, the optimization of the medium can also increase biomass. It was also found that full-strength MS supplemented with a lower concentration of PGRs has a great role in FC formation. The superior PGRs combination was found to be 2,4-D and kin (1 and 0.2 mg l^{-1}) which showed the most effective role in the FC formation. On the other hand, a protocol was developed to establish an efficient saffron CSC with higher biomass. In this protocol, an improved MS medium was developed, in which the total nitrogen was reduced to 30 µM and supplemented with 2,4-D (0.5 mg l^{-1}), zeatin (0.2 mg l^{-1}) and glutamine (10 mg l^{-1}). This protocol could successfully increase fresh cell weight from 0.2 to 1.2 g. In addition, it is suggested to investigate the production of saffron metabolites under CSC conditions.

Conflict of interest

The authors declare no conflict of interest to report.

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