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In Vitro Indirect Somatic Embryogenesis and Secondary Metabolites Production in the Saffron: Emphasis on Ultrasound and Plant Growth Regulators

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ABSTRACT

In the present study, the effects of ultrasound and plant growth regulators on in vitro callogenesis and secondary metabolites production in saffron calli were investigated. Accordingly, the saffron corms surface sterilized, sonicated and cultured on different concentrations of plant growth regulators (0.5, 1, 2 and 4 mg L^{-1} 2,4-D or NAA in combination with 0.5 and 1 mg L^{-1} Kin or BAP). The percentage of callus induction, callus yield (fresh weight) and embryogenic callus formation were recorded and secondary metabolites of calli were measured by UV/VIS spectrophotometer three months after culture. The results indicated that sonication of the saffron corm explants significantly increased the in vitro callus induction and growth. So, the highest callus induction (100%) and yield (4.68 g) was achieved with sonicated explants cultured on MS medium supplemented with 2 mg L^{-1} 1-naphthaleneacetic acid (NAA) and 0.5 mg L^{-1} kinetin (Kin). Somatic embryogenesis was significantly influenced by plant growth regulator regimes and the MS medium supplemented with 0.5 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) plus 0.5 mg L^{-1} 6-benzylaminopurine (BAP) and 0.5 mg L^{-1} NAA plus 0.5 mg L^{-1} Kin exhibited the highest percentage (75 and 72, respectively) of somatic embryogenesis. Secondary metabolite content of the callus cells was significantly different among the plant growth regulator regimes and the highest production of picrocrocin and safranal were occurred on the medium containing 0.5 mg L^{-1} 2,4-D plus 0.5 mg L^{-1} BAP and 1 mg L^{-1} NAA plus 1 mg L^{-1} BAP.

Keywords: *Crocus sativus* L; Saffron secondary metabolites; Sonication; Tissue culture

1. Introduction

Saffron, *C. sativus* L. from the genus Crocus and Iridaceae family, is one of the most important medicinal plants and valuable products of Iran. Saffron is currently being cultivated in many countries mainly Iran, Morocco, India, Greece, Spain and Italy (Ghorbani 2008). In the past years, researchers showed that the crocetin, crocin, safranal and picrocrocin, the most valuable secondary metabolites in the saffron, have anticancer properties, mainly on papilloma, squamous cell carcinoma, leukemia and soft tissue sarcoma (Chryssanthi et al 2007). Various carotenoids and unique compounds called apocarotenoids found in Saffron. This apocarotenoids are produced via oxidative tailoring of carotenoids (Auldridge et al

2006). Apocarotenoids are synthesized in a number of plants such as Maize, Tomato, Arabidopsis, Crocus and etc., but Crocus species is the only plants which synthesize crocin, picrocrocin and safranal in significant quantities (Liao et al 1999). The saffron apocarotenoids are formed by zeaxanthin cleavage followed by specific glycosylation steps (Rubio et al 2008).

Plant cell and tissue culture techniques offer effective approaches for improvement and propagation of plants such as saffron (Castellar & Iborra 1997). Recent fields of application of plant cell culture are the large scale production of secondary metabolites and other molecules of pharmaceutical interest, such as heterologous proteins from cell suspension cultures (Wilson & Roberts 2012). Plant cell growth and production of secondary metabolites affected by many factors in vitro, e.g. plant growth regulators (PGRs), which are one of the most important agents influence cell growth, differentiation and secondary metabolites formation in plants (Liang et al 1991). On the other hands, optimization of medium components and culture conditions is the critical factor in the callus induction, cell suspension culture establishment and growth, and metabolite synthesis in the plant cells.

Low energy ultrasound (US) acts as a physical stress, which influence the biological, biochemical and developmental processes of the plant cells (Teixeira da Silva & Dobranszki 2014). Cavitation and acoustic micro streaming produced by low-frequency ultrasound cause the modifications in the cellular ultrastructure, cell membrane permeability, enzyme permanency and finally cell development progress (Ward et al 2000; Guzman et al 2001). Regarding the wide pharmaceutical properties of saffron secondary metabolites and low productivity of dried saffron pistils (approximately 6-10 kg h^{-1}), in vitro plant cell culture techniques provide an attractive and reliable alternative source for production of crocin and its derivatives. Therefore, the main objectives of this study were: (1) set up an efficient protocol for callus induction and somatic embryogenesis by applying the PGRs and ultrasound treatment, (2) assessment of main secondary metabolites production in saffron callus under different regimes of PGRs.

2. Material and Methods

2.1. Plant material and sterilization

C. sativus L. corms were prepared from the Ghaen, South Khorasan Province, Iran (33°43′N 59°11′E) in June 2014. Corms of 1-2 cm in diameter were used as explant. The external sheath of corms which microorganisms ubiquitously found on them, were removed. Corms were thoroughly washed under running tap water for 30 min and surface sterilized using the following procedure; Corms treated by ethanol (70% v v^{-1}) for 90 s, washed one times with sterile distilled water (SDW), then immersed in commercial sodium hypochlorite (5% w v^{-1}) plus one drop of Tween-20 per 100 mL for 15 min. Corms were then thoroughly rinsed 3-4 times with SDW and cultured on the MS (Murashige & Skoog 1962) medium supplemented with different combinations of PGRs. All the disinfection steps were carried out in aseptic conditions in a laminar air flow cabinet.

2.2. Callus induction

In order to preparation of leaf explants, corms were cultured on the Hoagland solution for 10 days and then, new and fresh developed leaves were surface sterilized, and were cut into small fragments (0.5-1 cm).

To prepare corm explants, the sterilized corms were cut into two or more (depending on their size) equal parts with meristematic tissues attached to them. The leaf and corm explants were cultured on agar-solidified (0.8% w v^{-1}, Merck, Darmstadt, Germany) MS media supplemented with different concentrations of plant growth regulators (0.5, 1, 2 and 4 mg L^{-1} of 2,4-D and NAA in combination with 0.5 and 1 mg L^{-1} of Kin and BAP) and sucrose (30 g L^{-1}).

For assessing the effects of ultrasonic waves on in vitro callogenesis of saffron, the sterilized explants were sonicated for 5 min at 25 ºC in a bath sonicator (Bandelin electronic®, Germany) at 35 kHz and then cultured on MS medium supplemented with

aforementioned PGRs. Sonication of the explants were repeated every two weeks for 1 min, so that the cultured jars dipped in bath sonicator. Unsonicated explants were used as control.

Cultures were maintained in a growth room at 25±1 ºC, 16 h photoperiod, 700-800 lux of cool-white light intensity and were subcultured at monthly intervals. The percentages of callus induction and embryogenic callus formation, and callus yield (fresh weight) were recorded after three months.

2.3. Extraction and determination of secondary metabolites

Secondary metabolites of saffron callus were extracted by methanol (Lage & Cantrell 2009). Briefly, approximately 600-800 mg of callus samples were extracted with 8 mL of methanol, sonicated for 1 h and then stored overnight at 4 °C. The next day, the samples were sonicated again for an hour, and brought to initial volume with methanol. Samples were passed through 0.22 µm filter (Albet labscience, 0.22 µm pore size) using a sintered glass funnel and under vacuum. In order to prevent degradation of saffron secondary metabolites, the whole process was carried out in darkness and at room temperature.

The main secondary metabolites of saffron callus were determined according to ISO 3632 trade standard (ISO/TS 3632-2, 2003) method at 440, 330 and 257 (nm) wavelengths corresponding to the maximum absorbance of the coloring strength (crocin), the aromatic strength (safranal) and the bitterness (picrocrocin), respectively. The absorbance readings obtained using a UV/VIS spectrophotometer (SmartSpec Plus spectrophotometer, Bio-Rad, Hercules, CA, USA) and quartz cell of 1 cm path-length. The extracts were diluted 4-6 times in order to minimize derivations in Beer-Lambert's law (Lakowicz 2006). Value of each compound was calculated (for crocin, safranal and picrocrocin) using Equation 1 and 2 (ISO/TS 3632-2 2003):

$$E_{1cm}^{1\%} = \frac{D \times 10000}{m(100 - H)} \quad (1)$$

$$H = \frac{Initial\ mass - Constant\ mass}{Initial\ mass} \times 100 \quad (2)$$

Where; D is the absorbance at 257 nm (for the picrocrocin), 330 nm (for the safranal) and 440 nm (for the crocin); m is the mass of the callus (g) and H is the moisture and volatile content of the sample, expressed as a mass fraction. For determination of moisture and volatile contents, samples were maintained uncovered in an oven (103 ºC) for 16 h. The moisture and volatile matter content are calculated as a percentage of the initial sample using the Equation (2) (ISO/TS 3632-2 2003). The reported values are the average values of three replicates.

2.4. Statistical analysis

All data were analysed by one-way ANOVA followed by Duncan's multiple range test or least significant difference (LSD) mean comparisons. The experimental design was a factorial based on CRD with three replicates and 8-9 explants per replicate. All analyses were performed using SPSS ver. 16 (SPSS Inc, Chicago, IL.) and SAS Ver. 9 (SAS Institute, Cary, NC, USA) softwares and the graphs were produced using Microsoft Office Excel 2010. All values were presented as mean ± SE (Standard Error) with significance at $P \leq 0.05$.

3. Results and Discussion

3.1. Callus induction and somatic embryogenesis

Callus induction and formation was not occurred from the leaf explants (Figure 1a). In contrast, callus initiation and formation was occurred from buds and cut edges of the corm explants within two months (Figure 1b). The nonembryogenic calli was characterized by its yellowish color (Figure 1c). Data analysis indicated that percentage of callus induction and callus yield (fresh weight) were significantly ($P \leq 0.01$) influenced by PGRs and ultrasound treatments. Frequency of calli initiation ranged from 25% to 100%, depending on plant growth regulator combination and ultrasound treatment (Figure 2 and 3). In the treatments without ultrasound, the highest percentage of callus induction (100%) and callus yield (4.3 g per explant) was achieved on MS medium supplemented with 2 mg L^{-1} NAA and 0.5 mg L^{-1} Kin, which was significantly higher than those

Figure 1- Callus induction and embryogenesis from *Crocus sativus* L. corm explants culture on MS medium supplemented with 0.5 mg L^{-1} NAA and 0.5 mg L^{-1} Kin; a, leaf explants; b, corm explants; c, Nonembryogenic callus; d-f, somatic embryos at different stages of development (ME, matured embryo; GS, globular stage; HS, heart stage).

of other PGRs combination. While, the corm explants cultured on MS medium supplemented with 1 mg L^{-1} NAA plus 1 mg L^{-1} BAP or Kin and 4 mg L^{-1} 2,4-D plus 0.5 mg L^{-1} Kin exhibited lowest percentage of callus induction and callus yield in without sonication condition (Figures 2 and 3). In the study of Vahedi et al (2015) callus formation from saffron was successfully achieved by using a combination of 2.4-D and Kinetin hormones, which agreed with studies reported by Chaloushi et al (2007), Karamian & Ranjbar (2010) and Dalila et al (2013). They observed the highest frequency of callus induction (44%) on MS medium supplemented with 2.4-D (2 mg L^{-1}) and Kinetin (0.5 mg L^{-1}), culture from apical meristem. Darvishi et al (2006) also reported that a media containing NAA and BAP induced non-embryogenic calli; in contrast media containing 2.4-D and BAP had the best effect on induction of embryogenic calli in saffron.

As shown in Figures 2 and 3, stimulatory effects of ultrasound were different and varied in different PGRs treatments. Sonication of the explants were led to 2.0, 1.42 and 1.38-fold increase in callus induction on MS medium containing 1 mg L^{-1} NAA + 1 mg L^{-1} BAP, 1 mg L^{-1} NAA + 1 mg L^{-1} Kin and 0.5 mg L^{-1} 2,4-D + 0.5 mg L^{-1} BAP, respectively. While, sonication of explants did not influenced the callus induction on MS medium supplemented with 2 mg L^{-1} NAA + 0.5 mg L^{-1} Kin, which was exhibited 100% callus induction in the absence of ultrasound treatment. In contrast, sonication of explants reduced the callus induction on MS medium supplemented with 0.5 mg L^{-1} 2,4-D + 0.5 mg L^{-1} Kin (Figure 2).

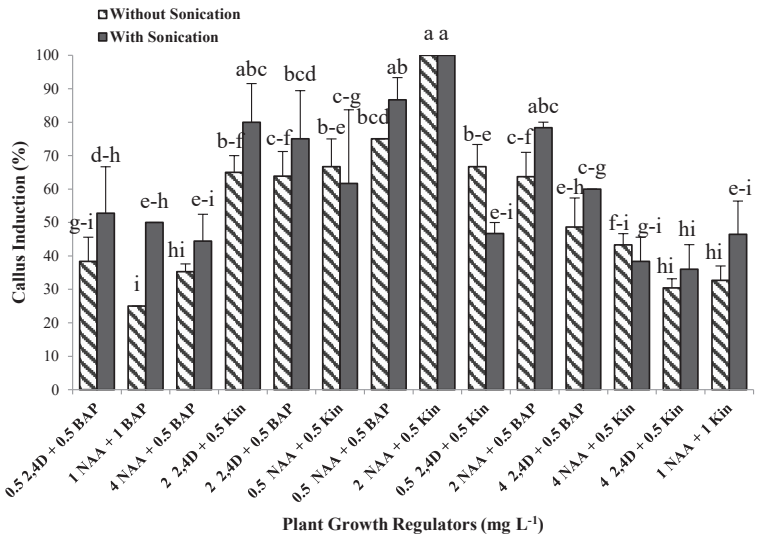

Figure 2- The effect of ultrasound on the callus induction from corm explants of *Crocus sativus* L. Each value represents the mean±SE of three replicates. Values with different letters are statistically different at P≤0.05

It has been shown in the past studies that ultrasound could be stimulate development in several plant species depending on its frequency and exposure time applied. Wang et al (1998) showed that growth rate of carrot (*Daucus carota* L.) cells in suspension culture was enhanced by about 5% while exposed to ultrasound (28 kHz) for 2 s, but exposure to US for 40 s reduced the growth rate by 57% relative to the control. The same results also were obtained in Liu et al (2003a) study. Liu et al (2003a) applied ultrasonic stimulation to rice (*Oryza sativa* L.) 'Nipponbare' cell culture with the same frequency (28 kHz). Results showed that cell growth was enhanced after exposure to 2 and 5 s. But cell growth and proliferation was inhibited when exposure time increased (30-120 s). In other study investigated by Wang et al (2004), Chrysanthemum callus sonicated with a frequency of 1.4 kHz twice daily for two 0.5 h periods. After 20 days, callus growth accelerated (unquantified) and differentiation of the shoots (28% higher shoot forming index than the control) stimulated. Low frequency ultrasound may vary the permeability of cell membranes (Rokhina et al 2009), subsequently may change the activity and the conformation of membrane-bound enzymes (Wang et al 2002; Liu et al 2003b) and thus may improve the transport processes in cells. Acoustic cavitation (the growth and collapse of pre-existing microbubbles under the influence of an ultrasonic field in liquids) is another consequence of ultrasound, which may influence plant metabolism (Rokhina et al 2009).

The growth regulators especially auxins and cytokinins play critical role in the control of plant cells division, growth and development, and a combination of auxins and cytokinins is mostly needed for reinitiate cell proliferation in tissue culture (Van Staden et al 2008). Plant species and genotypes show various in vitro growth responses to different types and concentrations of auxins and cytokinins (Guo et al 2009). As presented in Figure 3 (without sonication), although all combinations of PGRs combination successfully induced callogenesis from corms explants, however, various combinations of PGRs showed significantly different percentage of callus induction and callus yield. Generally, our findings suggested that combination of 2 mg L^{-1} NAA or 2,4-D with 0.5 mg L^{-1} Kin or BAP were more favorable for callus induction and growth. Castellar & Iborra (1997) reported that MS medium supplemented with 10 mg L^{-1} NAA and 5 mg L^{-1} BA was more favorable for callus induction

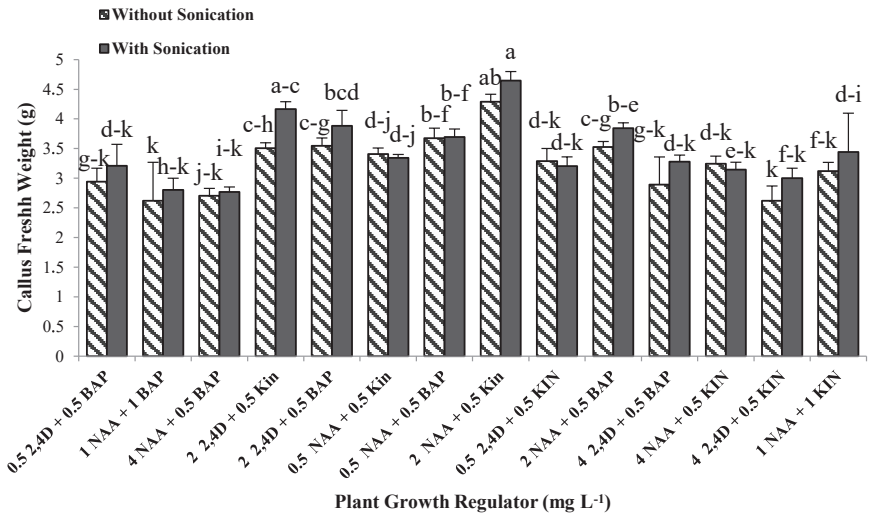

Figure 3- The effect of ultrasound on the callus fresh weight from corm explants of *Crocus sativus* L. Each value represents the mean±SE of three replicates. Values with different letters are statistically different at P≤0.05

and undifferentiated growth, while the MS medium containing 1 mg L^{-1} BAP and 1 mg L^{-1} NAA was the most appropriate for stigma differentiation. But, our results indicated that increasing NAA or 2,4-D levels to relatively high concentration (4 mg L^{-1}) led to reduced callus induction and growth. Recently, Verma et al (2016) reported the friable callus initiation in five Turkish *Crocus* species (*Crocus specious* ssp. *Specious*, *Crocus oliveri* ssp. *Oliveri*, *Crocus pestalozzae*, *Crocus abantensis*, *Crocus paschei*) within 20 days on the medium supplemented with 4 mg L^{-1} TDZ+4 mg L^{-1} NAA and 5% (w v^{-1}) sucrose. These inconsistencies could be explained by differences in the genotype of plant materials used in various studies, which can strongly affect the in vitro response of plant cell to PGRs (Venkatachalam & Jiayabalan 1997).

Our results demonstrated the sonication of corm explants stimulate the percentage of callus induction and callus yield of *C. sativus* L. even up to two-fold of without ultrasound treatments. But, the stimulus effects of ultrasound were varied depending on PGRs combination (Figures 2 and 3). The effectiveness of sonication depends upon the intensity, ultrasound frequency and exposure period (Rokhina et al 2009). The low-energy ultrasound (US), as a physical stimulus initiate a range of biochemical processes in the plant cells. These effects mainly rise from mechanical and cavitation properties of ultrasound irradiation, which significantly stimulate protein synthesis, cellular metabolic activity of enzymes in plant cells and protoplasts. Additionally, ultrasound cause transient pore formation on the cell wall and membrane and in enhanced plasma-membrane permeability that facilitate mass transfer and uptake of molecules from the medium and release of intracellular products by the cells (Dong et al 2002). The ultrasound treatment at 300 W for 5 min improved the conversion frequencies of protocorm like bodies (PLBs) to shoot in the *Dendrobium officinale* (Wei et al 2012). In Zare et al (2014) study, the combination of ultrasound (10 s) and L-tyrosine feeding (2 mM) significantly increased the production of thebaine in comparison to individual utilisation of 2 mM L-tyrosine and ultrasound (10 s).

Analysis of somatic embryogenesis (Figures 1d-f) data revealed significant differences ($P \leq 0.01$) between different PGRs. As shown in Figure 4, in the MS medium supplemented with 0.5 mg L^{-1} 2,4-D plus 0.5 mg L^{-1} BAP and 0.5 mg L^{-1} NAA plus 0.5 mg L^{-1} Kin, the most of explants and calli (75% and 72.22%, respectively) were produced somatic embryos, which was significantly higher than those of other treatments. Contrariwise, the explants cultured on medium containing higher level of auxin (4 mg L^{-1} 2,4-D or NAA) exhibited the lower somatic embryogenesis response compared to others. According to the past studies, decreasing concentration or complete removal of exogenous auxins has been reported to be essential for embryo maturation and further development (Sharifi et al 2012). Selection of embryogenic regions and transferring them into growth regulator free media caused to notable growth of embryogenic calli. It was recommended that division of the pre-embryogenic cells and their development into embryos is only resumed at lower auxin concentrations (Karamian & Ebrahimzadeh 2001). These findings are in line with our results that the highest somatic embryos were obtained in low concentrations of auxins and contrariwise the lowest percentage of somatic embryogenesis (8.33%) was obtained on MS medium supplemented with high level of auxin (4 mg L^{-1} 2,4-D and 0.5 mg L^{-1} Kin) (Figure 4). Somatic embryogenesis provides an effective approach for large-scale plant micropropagation, production of transgenic plants, artificial seed production and cryopreservation (Kita et al 2007). As shown in Figure 4, the effect of type of auxins (NAA or 2,4-D) and cytokinins (BAP or Kinetin) on somatic embryogenesis (%) were not significant, but concentration of both auxins and cytokinins meaningfully influenced the somatic embryogenesis of saffron corm explants. So that the efficiency of low and moderate concentrations of auxins (NAA or 2,4-D) in combination with BAP or Kin were significantly high and the maximum values of somatic embryogenesis belonged to the MS medium supplemented with 0.5 mg L^{-1} NAA or 2,4-D plus 0.5 mg L^{-1} BAP or Kin. It has been shown that expression of somatic embryogenesis influenced by different factors including plant species and

cultivar, medium composition (especially by types and concentrations of PGRs), environmental and physiological conditions of the donor plant (Bajaj 2013). Karamian (2005) were obtained the best embryogenesis rates in the presence of 4 mg L^{-1} kinetin and 1 mg L^{-1} 2,4-D, as approximately 30% of calli formed on this medium produced somatic embryos.

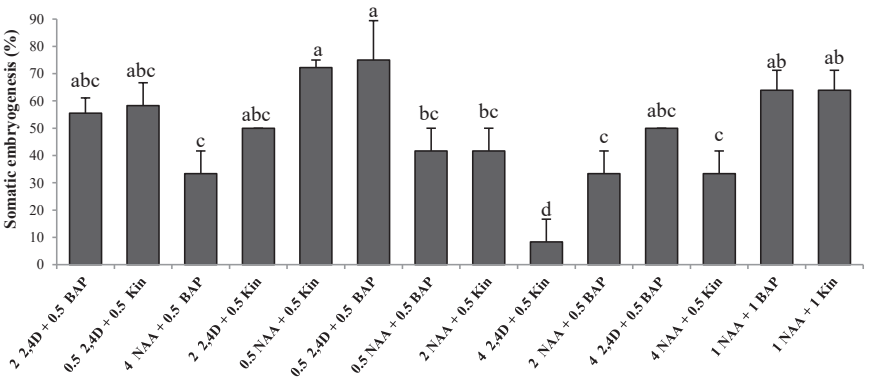

Figure 4- Effect of various concentrations of PGRs on percentage of *Crocus sativus* L. explants showing somatic embryogenesis response. Each value represents the mean±SE of three replicates; values with different letters are statistically different at P≤0.05

3.2. Secondary metabolites

The effects of different concentration of auxins (2,4-D and NAA) and cytokinins (BAP and Kin) on picrocrocin, safranal and crocin production were shown in the Figure 5. As shown in the Figures 2 and 3, although the combination of NAA (2 mg L^{-1}) and Kin or BAP (0.5 mg L^{-1}) provided optimal condition for callus growth and proliferation, the highest picrocrocin production and accumulation was obtained from the calli grown on MS medium supplemented with 0.5 mg L^{-1} 2,4-D plus 0.5 mg L^{-1} BAP. Safranal content of the calli grown on MS medium supplemented with 1 mg L^{-1} NAA and 1 mg L^{-1} BAP or 1 mg L^{-1} Kin were significantly higher than those of others. As shown in Figure 5, picrocrocin and safranal production and accumulation in the saffron calli decreased significantly with increasing the concentration of NAA from 1 mg L^{-1} to 2 and 4 mg L^{-1}. The lowest content of the crocin was obtained in the MS medium supplemented with 2 mg L^{-1} NAA and 0.5 mg L^{-1} BAP. It has been shown that production of secondary metabolites in the plant cells influenced by many environmental factors (such as temperature, humidity, CO_2, photoperiod and light intensity) and medium composition (mainly by PGRs, type and concentration of carbohydrate and nitrogen source) (Akula & Ravishankar 2011). In recent years, various studies have been conducted to establish practical approaches to enhance the yield of secondary metabolites under in vitro conditions. In contrast, there are very few reports on the effects of culture conditions and PGRs on the secondary metabolites (including crocin, picrocrocin and safranal) production on the saffron callus cultures. In the present study, the experimental data supported the speculation that the amount of safranal, crocin and picrocrocin in the saffron calli are dependent on type and concentration of PGRs. So that, our data revealed that the low or moderate levels of PGRs are optimal for the production and accumulation of secondary metabolite in the saffron callus (Figure 5). In Liu et al (2002) studies, best results for promotion of saffron callus growth and crocin biosynthesis achieved by NAA and IAA application, respectively. Their findings showed that IAA (4 mg L^{-1}), GA_3 (2 mg L^{-1}) and uniconazole (S-07) (1.25

mg L^{-1}) remarkably enhanced the crocin content in calli. They also found that NAA (2 mg L^{-1}) promoted the growth of saffron callus but had no advantage and may inhibit crocin synthesis whereas uniconazole (1.25 mg L^{-1}) had the opposite effect and the growth of calli with S-07 was significantly inhibited. In contrast, GA_3 promoted both growth and synthesis. Plant growth regulator plays important role in secondary metabolites accumulation in a plant cell and tissue culture studies (15). It has been that auxin and cytokinin individually or in combination significantly could alter both the growth and secondary metabolite accumulation in cells cultures (Dicosmo & Towers 1984). Sahai & Shuler (1984) showed that NAA or IAA enhanced the production of nicotine in suspension culture of *N. tabacum.* Similarly results obtained in Coste et al (2011) study in which investigate the effects of plant growth regulators (BA, Kin, 2iP, TDZ and NAA) and two elicitors (jasmonic acid and salicylic acid) on accumulation of hypericins (hypericin and pseudohypericin) and hyperforin in shoot cultures of *Hypericum hirsutum* and *H. maculatum*. Their findings revealed that culture of shoots on MS medium supplemented with BA (0.4 mg L^{-1}) or Kin (0.4 mg L^{-1}) enhanced production of hypericins and hyperforin in *H. maculatum* and *H. hirsutum*, respectively. Carmona et al (2007) identified the flavonoid fraction in saffron spice using of LC-DAD/MS/MS ESI. They also analysed is there if differences in the flavonoid contents of samples from different geographical origins that could be used as biomarkers for the determination of saffron origin? They found five kaempferol derivatives in which kaempferol 3-sophoroside contents of the saffron samples could clearly separate different geographical origins.

4. Conclusions

In conclusion, present study showed that ultrasound together with PGRs extremely affected the saffron callus and cell growth in solidified MS. Based on the results, it seems that high concentration of auxins inhibit both cell growth and secondary metabolite production in saffron calli. The results suggested that maximum callus growth and valuable secondary metabolites production in the saffron in vitro cultures could be achieved in the moderate concentrations of PGRs.

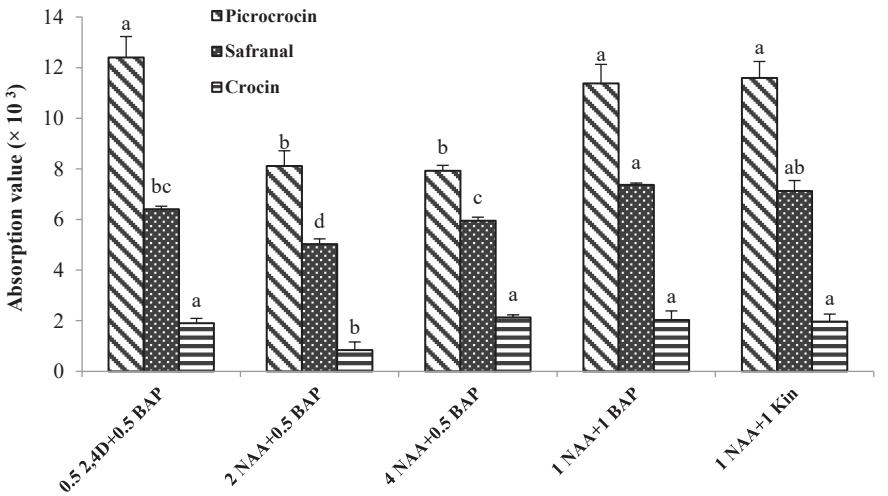

Figure 5- Comparison of $E^{1\%}_{1cm}$ values for picrocrocin, safranal and crocin under different treatment extracted by ISO/TS 3632-2 procedure in *Crocus sativus* L. Each value represents the mean±SE of three replicates. Values with different letters are statistically different at P≤0.05

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