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In Vitro Callus Induction of Saffron (Crocus Sativus L.)

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Abstract:- Saffron (Crocus sativus L.) belongs to Iridaceae family. The dark red-dried stigmas of the plant, which is called saffron, has special economic value and is the most expensive spice in the world. Considering its significant characteristics such as pleasant color, taste and aroma, it has been used as a flavoring agent and pharmaceutical plant for many years. The content of secondary metabolites of saffron due to its medicinal properties attracts attention. Saffron is propagated through its corms by traditional ways, but it is very costly. Hence, at present, the cultivation of plant tissue culture technique is one of the suggested ways to solve this problem. By using plant tissue culture techniques, the aim of this study was to use various compositions of plant growth regulators, as well as the use of carrot juice as a substitute of plant growth regulators for callus induction in in vitro conditions. The study findings showed that cell differentiation and embryo development of saffron was successful from induced callus through culture of various explants on media containing plant growth regulators and could be one of the short propagation methods for saffron. However, in the media containing carrot juice, no callus or embryos development was observed.

Keywords:- *Saffron (Crocus sativus L.), Plant Tissue Culture, in vitro, Callus Induction.*

I. INTRODUCTION

The Crocus genus is a member of the large family of Iridaceae, including more than 80 species, with about 30 species cultivated throughout the world (Lagram et al., 2016). Among the species of Crocus, Crocus sativus (saffron) is an excellent species. Since ancient times, dried stigmas of saffron flower have been known for their color and medicinal properties. Saffron contains more than 150 volatile and nonvolatile compounds, including quality involved three main secondary metabolites; crocine, picrocrocine and safranal, which are responsible for its color, taste and aroma respectively (Fahim et al., 2012). Due to its color, taste, aroma and medicinal properties, saffron is used in the food, cosmetics, perfumery and dye industries (Lagram et al., 2016). Saffron is also used to treat various diseases such as gastric disorders, colic, cough, insomnia, chronic uterine bleeding, vertigo, colds, asthma, cardiovascular disorders, and also as the anti-depressant (Jalal et al., 2015). Anti-diabetic, anti-seizure, anti-depressant, anti-inflammatory, and antioxidant activities of saffron have been shown in various research conducted (Shah et al., 2017).

Economically, saffron is the most expensive spice; the cost of one kilogram saffron is about $ 2,000. Iran and Spain are the largest producers of this product, which meet over 80% of global saffron production (Vermaa et al., 2016). Saffron is also cultivated in Italy, Greece,Turkey, Pakistan. In recent years, saffron has been cultivated in Afghanistan, which is considered one of the largest saffron producing countries. Iran is currently the first producer of saffron, followed by other countries such as Spain, Afghanistan and India. Afghanistan is ranked fourth among saffron producing countries with about 6 tons of saffron production (Aslami, Fifth National Conference on Saffron, 2017). In the Mediterranean regions, saffron is produced in other countries such Greece, Morocco, Italy, Turkey, France, Switzerland, Pakistan, Azerbaijan, China, Egypt, the United Arab Emirates, Japan and Australia (Lagram et al., 2016). It is worth mentioning that Afghan Saffron Company, a processing company, received the first place in the world for producing quality saffron from the International Taste and Quality Institute (iTQi) (http://masnad.af/2017/02/24/afghan-saffron-ranked-the-best-in-the-world/).

The natural growth rate of saffron is low, so this plant is mainly cultivated in vegetative manner, which is not economic due to saffron corms' cost. Therefore, in vitro regeneration techniques can be the alternative way for propagation (Mir et al., 2014). The techniques plant's organ, tissue and cell culture for the production of biomedical products have been the object of interest for research since the late 1950s (Escribano et al., 1999). Plant culture techniques in artificial culture media, in controlled containers under appropriate environmental conditions are used for the maintenance and growth of plant tissues, or organs. Parts of the plant which used for culture are called the explant. The purpose of micropropagation is to produce a large amount of seedlings of the same genotype with natural growth and development in the shortest possible time and at the lowest cost. Micropropagation is now expanding in horticulture, agriculture and forestry. The artificial culture media contain plant hormones or Plant Growth Regulators (PGR) and the essential nutrients for plant growth. Different groups of PGR have certain functions which are used in different combinations and quantities for different purposes and in different artificial containers. In some cases, extract of yeast, coconut milk and extract of plants can also play the role of PGR (Jalal et al., 2015). In study, hippocotyl plants were cultured on MS medium containing 2.5 mg / L BAP and 2.5 0.1 mg/L NAA as well as natural supplements such as 20% coconut juice,

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20% coconut milk, 10% grind spinach leaves, 10% crushed potatoes, 10% grind carrots, 5% rice flour, 10% green peas, 10% pumpkin, 10% banana, 10% orange and control - Without any suplement- (Manawadu et al., 2014).

There are a number of reports on *Crocus in vitro* propagation. Since crocus varieties are cultivated in many countries, it is necessary to have a suitable *in vitro* culture system. Therefore, improving the tissue culture protocol *in vitro* is important to maintain the mass of the germ plasm, as well as its commercial distribution. But so far no research has been conducted on the *in vitro* culture of Afghan saffron, and there is also no report on the use of plant extracts in the artificial media instead of PGR for saffron. In this article we describe a simple and very effective protocol for *in vitro* callus induction of Afghan saffron. Also, for the first time different percentages of carrot juece used instead of plant hormones in comparison with the media containing plant hormones to evaluate its effects on the growth of callus and organs.

II. MATERIAL AND METHOD

Crocus sativus corms were provided in cooperation of "Herat Saffron Company" (changed to: Afghanistan Red Gold Company) from saffron farms of Herat province of Afghanistan and transferred to Izmir, Turkey for conducting research. First, the corms cultivated in Ege University green house for obtaining other explants. Afterward both the corms and the other parts of saffron, which were grown from saffron corms in greenhouse condition, used as explant. The corms, mature and immature flower parts, and green leaves cultured on artificial nutrient media (Figure 1).

MS (Murashige & Skoog (1962)) is the basic culture media used for all saffron explants, containing different groups of PGR in different values. These media contain 50% of sucrose in difference from the basic MS medium which containing 30% of sucrose. In addition, the pure carrot juice in different percentages was used in MS media to evaluate the role of the carrot extract in growth of callus and embryo and plant organs instead of PGR. The media were coded for ease of work and evaluation (Table 1).

Media Code	Basic Media	2,4-D (mg/L)	BAP (mg/L)	IAA (mg/L)	Carrot juice %
*SK	**MS	1	1	-	-
SH1	MS	-	-	-	5
SH2	MS	-	-	-	10
SH3	MS	-	-	-	20
SH4	MS	-	-	-	35
SÇ	MS	-	1	0.1	-

Table 1:- Culture media codes, the contents of PGR and carrot juice with different amounts
*containing 50% sucrose in contrast of MS basic media which contains 30% sucrose **Murashige & Skoog (1962)

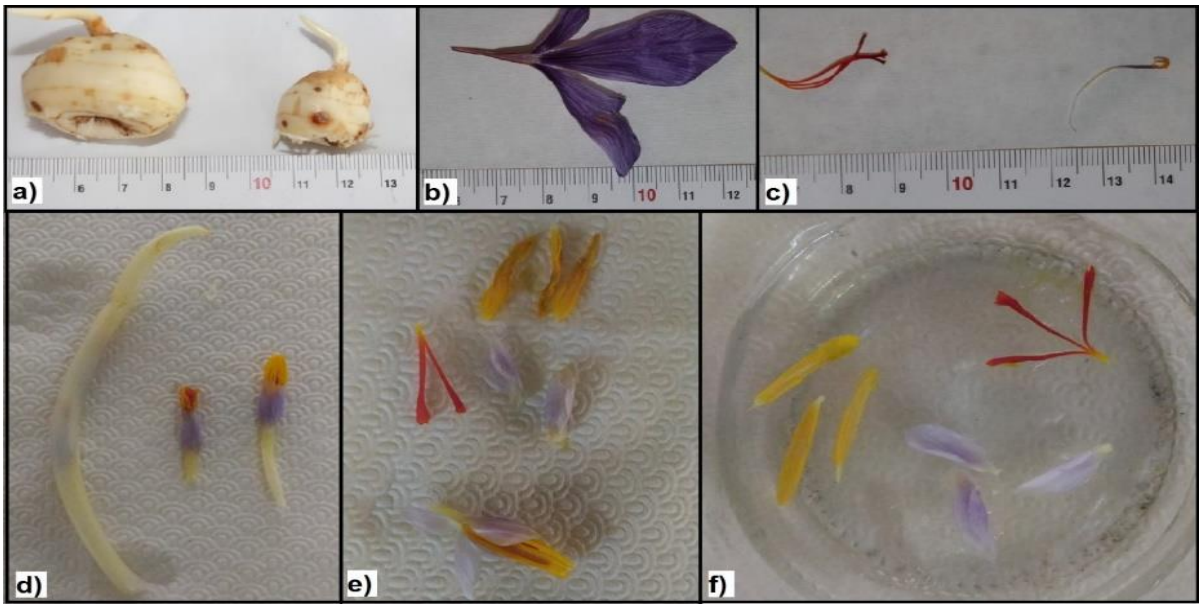

Fig 1:- Different parts of saffron for culture: a) corms, b) petals, c) stigma and style, d) saffron corm buddings, each containging two young flowers, e) immature flowers of saffron, f) immature flower parts of saffron on culture media

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In order to carry out the culture, first the laminar air flow cabinet turned on about 15 to 20 minutes before starting the work. At the same time, the UV lights also turned on to eliminate the contaminations. Then, the cabinet surface cleaned with 75% ethanol. The instruments, such as pens, scalpel, culture media and other items of necessity, which were previously sterilized at 121 °C for 20 minutes in autoclave, were placed in the laminar and turned on the UV light for 15 minutes. Subsequently, we subjected the surface sterilization of the saffron explants inside the laminar. Different methods used for sterilization of saffron corms and the best method which could sterile saffron corms from contaminations was the method described by Vermaa et al. (2016), followed by Povidone-iodine solution 1% for 5 minutes before using Alcohol 75%, the same procedure was continued during the study for corms sterilization.

Explants from flower parts were sterilized only by 75% alcohol for 30 seconds for one minute. After sterilization, saffron corms cut and cultured on MS nutrient media supplemented by 1 mg / L BAP and 1 mg / L 2,4-D for callus induction. This media containing 50% sucrose in contrast to the basic MS media which contains 30% sucrose. In addition MS culture media containing 5, 10, 20 and 35% pure carrot juice was used for saffron corms and other saffron explants being cultured such as flower parts (matured and immature petal, stigma and style), and green leaves, which were grown on greenhouse conditions for callus induction. The petals were also cultured on MS media supplemented by 1 mg / L BAP and 0.1 mg / L IAA. All culture media containing different saffron explants were kept under 2400 lux, 16 hours of light and 8 hours of dark photoperiod conditions. Some parts of the saffron on the media are shown in Figure 2.

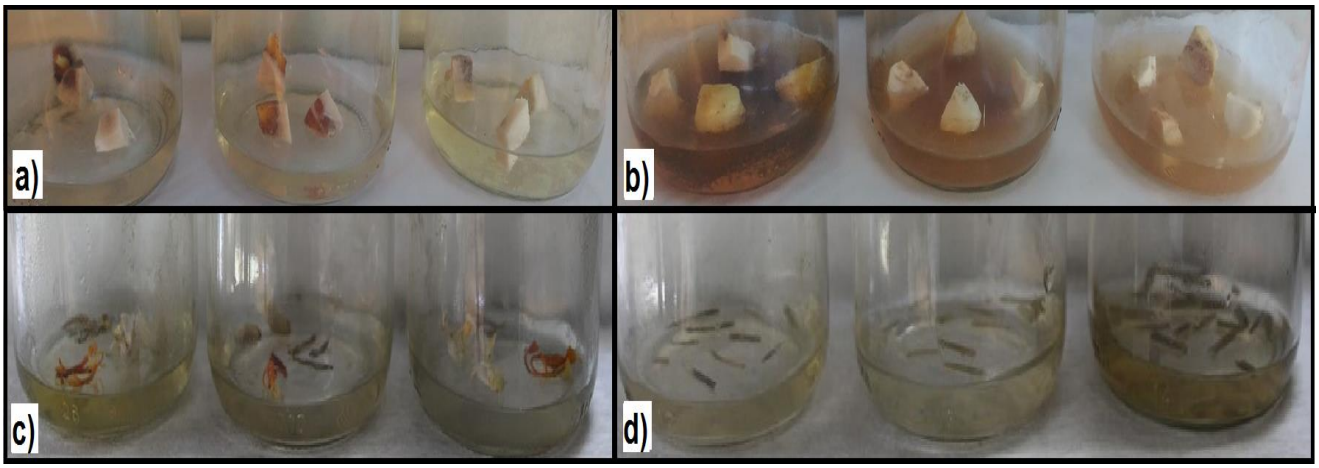

Fig 2:- Different parts of saffron; a) corms on SK media, b) corms on SH1, SH2, SH3, SH4 media, c) flower parts on SÇ media, and d) leaves on SK media

III. RESULTS

After four and six weeks, different explants of saffron that were cultured for purpose of obtaining callus, the development of callus were observed only from saffron corms cultured in tubes and immature stigmas (Figure 3, A and B), (Table 2). In order to grow callus from saffron corms, the culture medium containing 1mg / L BAP, 1mg / L 2,4-D and 50% sucrose was determined the best (17.6%), which was used for subsequent cultures (Table 2). After 12 weeks of culture, development of embryo (17.6%) was observed from callus obtained from the saffron corms, means that development of embryo observed in all callus forming corm explants (Figure 3, A). It should be noted that the growth of callus and embryo from saffron corms was observed only in the tubes, while the media containing the same contents in the jars did not show any positive results, which indicates the role of container shape in the development of plant callus and organs. However, the best media for callus induction was MS media supplemented by 1 mg / L BAP and 0.1 mg / L IAA by showing 42.9% of callus growth (Figure 3, A and Table 2). It should noticed that media containing carrot juice did not show any callus, embryo or organ development (Figure 3, B).

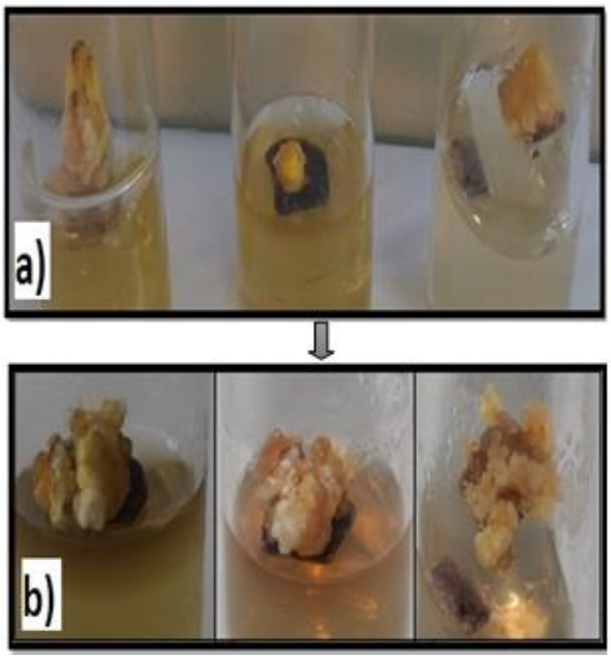

Fig 3:- A. Observe calluses and embryos of saffron corms: a) After 7 weeks, b) after 12 weeks

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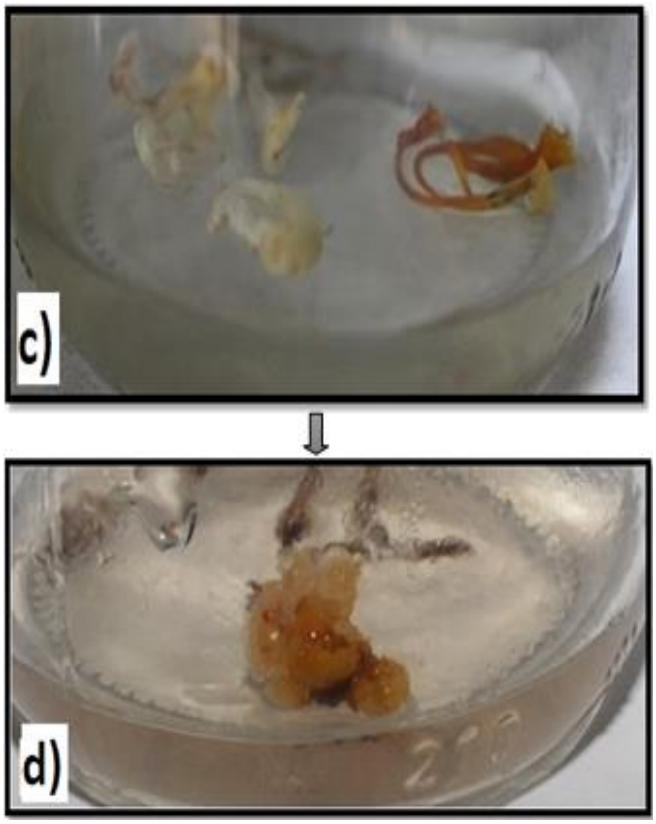

Fig 3:- B. Growth of callus from saffron immature stigmas: c) After 4 weeks, d) after 10 weeks

IV. CONCLUSION

Among the saffron explants cultured on different nutrient media, callus induced and developed only from corms and immature stigmas on MS media supplemented by 1mg / L BAP, 1mg / L 2,4-D (Tubes) and MS media supplemented by 1 mg / L BAP and 0.1 mg / L IAA. Globular embryos also developed from callus of saffron corms. Callus growth and embryo development had seen only from saffron corms which were cultured on tubes, which indicates the role of type of container in developing different plant species in in vitro plant tissue culture. In a study conducted by Jalal et al. (Jalal et al., 2015), the culture media containing extract willow bean shows successful results for saffron callus induction. However, in the current study, the MS media containing the pure carrot juice, where only saffron corm were cultured on them, did not show any positive results for the growth of callus, embryos or plant organs.

Although many studies have been conducted on the in vitro tissue culture of saffron, with postitive results (Yasmin et al., 2013, Simona et al. 2013, Mir et al., 2014, Jalal et al. 2015, Lagram et al., 2016, Vermaa et al., 2016), but in this study, the callus and embryo development protocol of Afghan Saffron is presented for the first time. One of the results of this study is that by obtaining cells from callus and using the cells as bioreactors, a new protocol for the producing larger amounts of saffron quality involved secondary metabolites in subsequent studies is suggested.

Nutrient Media	Explant type	Culture vessel type	Explants formimg callus (%)	Explants formimg embryo (%)
SK	Corms	Tube	17.6	17.6
SK	Corms	Jar	0.0	0.0
SH1	Corms	Jar	0.0	0.0
SH2	Corms	Jar	0.0	0.0
SH3	Corms	Jar	0.0	0.0
SH4	Corms	Jar	0.0	0.0
SK	Leaves	Jar	0.0	0.0
SÇ	Matured stigma	Jar	0.0	0.0
SÇ	Matured style	Jar	0.0	0.0
SÇ	Matured petal	Jar	0.0	0.0
SÇ	Immatured stigma	Jar	42.9	0.0
SÇ	Immatured style	Jar	0.0	0.0
SÇ	Immatured petal	Jar	0.0	0.0

Table 2:- Percentages of developed Callus and embryo from the components of saffron cultured on different culture media

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