Activated charcoal improves growth of *F. imperialis* propagated by indirect organogenesis

Nigar SAEED [1,2*] - Sabahattin CÖMERTPAY[3]

1: Department of Ornamental Plants, Bakrajo Technical Institute, Sulaimani Polytechnic University, Sulaymaniyah, Iraq*; E-mail: nigarsabr2011@gmail.com*

2: Department of Bioengineering and Sciences, Institute of Science, Kahramanmaras Sutcu Imam University, Çevreyolu Blv. No: 251/ A 46040 -Onikişubat/Kahramanmaraş Turkey

3: Department of Agricultural Biotechnology, Agricultural Faculty, Kahramanmaras Sutcu Imam University, Çevreyolu Blv. No: 251/ A 46040 -Onikişubat/Kahramanmaraş Turkey; sacomertpay@gmail.com

Abstract: *Fritillaria imperialis* L production is a challenge for the producers since its regeneration from seeds needs several years. In vitro micropropagation is considered the most proper technique to guarantee a rapid, effective and healthy regeneration. In vitro culture effects of different doses (0.0, 0.5, 1.5, 2.5 g/L) of activated charcoal (AC), and three levels (0.0, 1.5, 1.75 mg/L) of 6-Benzylaminopurine (BAP) on the growth of *F. imperialis* plantlets were determined. The growth medium contained same concentrations of auxins (0.8 mg/L NAA and 0.4 mg/L IAA) for all treatments. The number of shoots, roots, and leaves in regenerated plantlets were increased by adding 2.5 g/L of AC in growth medium containing 1.75 mg/L of BAP. No effects of different cytokinin concentrations were observed on shoot length but added AC significantly improved the shoot length all amounts studied. High concentration of BAP (1.75 mg/L) gave adverse impact on root length in all treatments, but 1.5 g/L AC enhanced the root length and better interacted with cytokinin hormone at 1.5 mg/L level.

Keywords: Crown imperial, activated charcoal, micropropagation, indirect organogenesis

Introduction

Fritillaria imperialis L. (Liliaceae), widely known as crown imperial or Tears of Mary because of great drops of nectar at the petal base, is a perennial plant with ornamental and cut flower use. Besides, it is important for medicine because it contains steroidal alkaloids with various pharmaceutical properties (Gao et al., 1999; Perry and Metzger, 1980; Wang et al., 2005). The species of the Fritillaria genus are distributed in different parts of the world including Turkey, Iraq, Iran, Asia Minor, South Asia, South Eastern Asia and some parts of USA. In the natural habitat, a full-sized Fritillaria needs nearly five years to grow from the seed. On the other hand, the natural propagation rate of most geophytes is relatively low. This often hampers the large scale cultivation of these plants. Since it has small numbers of scales, the amount of meristematic cells in the plant is rather restricted. In vitro propagation methods are essential components of plant genetic resources management and they are becoming increasingly important for conservation of rare and endangered plant species (Almeida et al., 2005; Bhatia et al., 2002). The application of tissue culture techniques may allow rapid and large-scale propagation of uniform plants for field condition. Thus, other techniques such as vegetative propagation and micropropagation were used. On the other side, wild population of *F. imperialis* is highly heterozygote and non-uniform, so for the conservation of different *F. imperialis* populations, the regeneration pathway must be highly genotype-independent and applicable for a wide number of genotypes. Direct organogenesis pathways (regeneration without crossing callus) and indirect organogenesis pathway (regeneration with crossing callus) have been applied for overcoming genotype-dependency and retaining long-time to totipotency in cumin (Ebrahimie et al., 2006b).

In plant tissue culture, activated charcoal (AC) is widely used to stimulate rooting of

micropropagated plantlets, since it enhances the adsorption of metal irons, vitamins, plant growth regulators, and darkening the media (Dumas and Monteuuis, 1995). Moreover, AC adsorbs harmful substances produced either by the media or the explant (Fridborg and Eriksson, 1975; Fridborg et al., 1978).

One of the most important factor in vitro culture especially in proliferation stage is cytokinin hormone. It is well known that cytokinins play multiple roles in the plant development such as promotion of cell, division and cell expansion and protein synthesis stimulation in plant. The present study investigates effects of different doses of activated charcoal AC, different concentrations of 6-Benzylaminopurine (BAP), and their interaction on growth of in vitro micropropagted *F. imperialis*.

Materials and methods

F. imperialis wild grown bulbs obtained from a free trader in Adiyaman were used as plant material. Murashige and Skoog (Murashige and Skoog, 1962) (MS) basal medium was used in this experiment. The medium was gelled with 5.5 g/L agar plant (Duchefa Biocheme) in addition to 30 g/L sucrose as energy and carbohydrate source. The pH of the medium was adjusted to 5.5±0.9 before adding agar and prior autoclaving. After that, the medium was put in autoclave at 121 ^{0}C for 15 minute sterilization process. The cultures were saved in a growth room at 25±2 ^{0}C with 65% relative humidity, under white fluorescent light for 16h light photoperiod.

The bulbs were washing thoroughly under running tap water to remove the soil, dead parts and insects on the surface, and then, they were rinsed three times with distilled water as well, then after bulbs were cut to smaller pieces. Afterwards, these pieces were pre-treated with 1% (w/v) sodium hypochlorite with Tween-20 for 15 minutes. The prepared cuts were washed extensively with sterile water more than 3- 4 times. This process was completed under controlled and sterilized conditions. These surface sterilized pieces were cut at 1.5 cm width and 2 cm length, then after cultured on MS medium containing 0.125 mg/L Thidiazuron (TDZ).

From the generated callus, medium size pieces ‘2cm*2cm’ were selected and cultured in MS supplemented with different doses (0.0, 0.5, 1.5, 2.5 g/L) of AC, and three levels (0.0, 1.5, 1.75 mg/L) of BAP.

Statistical analysis: Statistical analyses were performed using the software IBM SPSS Statistics for Windows, Version 22.0. Analysis of variances was done by two way ANOVA to separate the activated charcoal effect, hormones effect, and their interaction. Tukey’s tests were performed for every possible pairwise comparison.

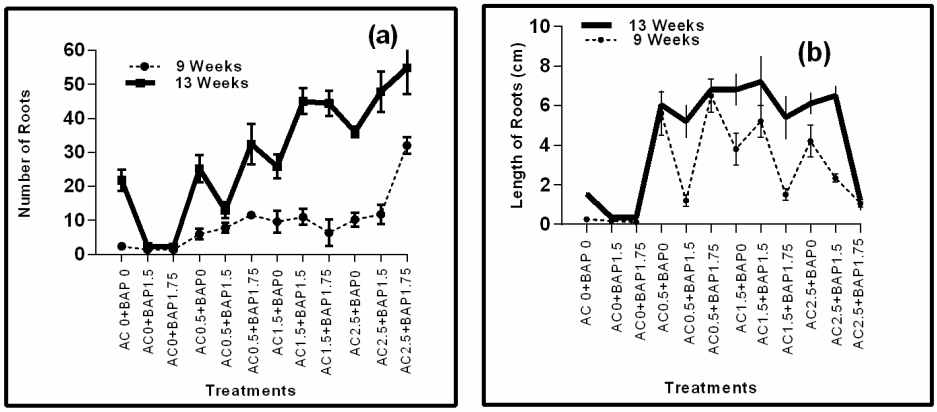
Results and discussion

In this study, effects of 0, 0.5, 1.5 and 2.5 g/L of activated charcoal (AC) alone or combined with three concentrations (0.0, 1.5, 1.75 mg/L) of 6-Benzylaminopurine (BAP) on growth parameters were evaluated on *Fritellaria imperialis* plantlets, cultured by indirect organogenesis. Number and length of the roots were observed after 9 and 13 weeks of treatment.

 DOI: 10.18380/SZIE.COLUM.2017.4.1.suppl

Adding activated charcoal to the growth media without cytokinin hormone increased the number in a dose-dependent manner reaching 10.2 and 36.0 at the 9th and 13th weeks of growth, respectively (Figure 1).

Of all conditions studied, the greatest increase in number of roots was observed when explants were grown in a medium containing the highest dose of AC (2.5 g/L) with the highest concentration of BAP (1.75 mg/L) for both 9 (32.0±2.5) and 13 (54.8±7.6) weeks (Figure 1). This observation might be related to the ability of activated charcoal to



adsorb all compounds in explants and media, also continuing division of the cells in the mersitematic region (Nhut et al., 2001).

Figure 1. Number of roots, and length of roots of F. imperialis plantlets, after 9, and 13 week. (a) Number of roots. (b) length of roots in centimetre. Vertical bars represent standard deviation (n=5).

On the other hand, the lowest numbers were counted in the treatments with no AC and higher concentrations of BAP; 1.38±0.5 and 2.20±0.8 for 9 and 13 weeks, respectively. This possibly related to the present of phenolic compounds and toxic brown pigments in the growth medium, as it is also reported by Fridborg et al. (1978), who found phenolic compounds and other metabolites in a medium without activated charcoal resulted in embryogenesis and morphogenesis inhibition.

Despite, differences within the same treatment, adding AC to the medium did positively affect the length of roots reaching the highest value 7.2 cm in ($AC_{1.5}$, $BAP_{1.5}$) treatment at week 13, meanwhile raising the AC dose to 2.5 g/L did not enhance the root length furthermore and gave an adverse effect especially when combined with 1.75 mg/L BAP. Excessive doses of AC in growth media can lessen its adsorption capacity due to many factors such as density, purity of the activated charcoal and the pH of the MS media (Druart and Wulf, 1993).

In contrast to our results, Rahimi et al. (2014) found no difference between treatments with or without activated cahrcoal, when they added 1g/L activated charcoal to 1/2 strength MS media for *F. imperialis.*

As it is shown in (Table 1), the superior dose of AC (2.5 g/L) along with a moderate concentration of BAP (1.5 mg/L) gave the highest number of shoots (32.4±5.0 and

43.2±3.1) after 9 and 13 weeks, respectively. The lowest number of shoots (10.4±2.7) were noticed with no activated charcoal at the same concentration of BAP in 9 weeks. In week13, however, the lowest number of shoots were observed (11.6± 2.07) only when no BAP or AC were added to the media.

The fact that a similar observation was also made for shoot numbers, which showed its highest numer at the highest concentration of AC, might be another evidence to support that the use of activated charcoal in the growth media stimulates and feeds the tissue more effectively, which in turn helps in modification and differentiation of the explant (Thomas, 2008).According to (Pan and Staden, 1998), AC can inhibit the toxic effect of phenolic components and help plant parts to uptake the nutritional compounds from the media, resulting in better growth.

Shoot length appeared to be affected greatly by both BAP and AC concentrations, and their combination providing us with various results. For samples plantlets treated for 9 weeks generated the longest shoot (1.12±0.10) at moderate concentrations (1.5 g/L of AC and 1.5 mg/L BAP). When the duration of the treatment was extended to 13 weeks, the longest shoot (1.50±0.12) was generated from the sample treated with relatively low concentration of AC (0.5 g/L) and the highest concentration (1.75 mg/L) of BAP (Table 1). In our study, higher concentrations of hormone affected shoot formation possibly related to the physiology and the multiformity of plantlets and the synthesis of endogenous hormones in the plantlets, different endogenous hormones of explants may differ in their ability to react to exogenously applied plant growth regulators (Winkelmann and Serek, 2005).

Table 1. Number of shoots, and length of shoots of F. imperialis plantlets, after 9, and 13 weeks. Means with different letters are significantly different at ($p<0.05$), determined by Tukey's HSD test (Mean ± SD, n= 5). Capital letters represent AC effect, small letters represent BAP effect.

AC g/L	BAP mg/L	Number of shoots		Length of shoots	
		9 weeks	13 weeks	9 weeks	13 weeks
AC_0	Cyt_0	10.6^{Aa} ±2.4	11.6^{Aa}± 2.07	0.25^{Aa}±0.07	0.90^{Ab}± 0.25
	$Cyt_{1.5}$	10.4^{Aa} ±2.7	11.8^{Aa}± 3.11	0.32^{Aa}±0.08	0.38^{Aa}± 0.17
	$Cyt_{1.75}$	12.8^{Aa} ±4.4	12.2^{Aa}± 3.11	$0.40A^{Bb}$±0.18	0.40^{Aa}± 0.18
$AC_{0.5}$	Cyt_0	11.8^{Aa} ±3.3	33.6^{Bb}± 2.3	0.96^{Ca}±0.05	$1.1A^{Ba}$± 0.10
	$Cyt_{1.5}$	12.8^{Aa}±1.6	23.4^{Ba}± 1.1	1.02^{Ba}±0.10	1.22^{Ba}± 0.16
	$Cyt_{1.75}$	23.4^{Bb}±3.9	31.8^{Cb}± 1.6	0.90^{Ba}±0.07	1.80^{Cb}± 0.27
$AC_{1.5}$	Cyt_0	13.8^{Aa}±3.3	14.00^{Aa}±6.8	0.78^{BCa}b±0.3	1.32^{Ba}±0.39
	$Cyt_{1.5}$	26.4^{Bb}±4.2	31.20^{Cc}±2.1	1.12^{Bb}±0.10	1.50^{Ca}±0.12
	$Cyt_{1.75}$	15.0^{Aa}±1.5	23.60^{Bb}±2.0	0.50^{Aa}±0.10	1.24^{Ba}±0.25
$AC_{2.5}$	Cyt_0	15.0^{Aa}±2.2	33.8^{Ba}± 1.9	0.48^{Ba}±0.08	1.62^{Bb}± 0.43
	$Cyt_{1.5}$	32.4^{Bc}±5.0	43.2^{Db}± 3.1	0.98^{Bb}±0.04	1.10^{Ba}± 0.10
	$Cyt_{1.75}$	26.0^{Bb}±2.9	36.2^{Ca}± 4.0	0.90^{Bb}±0.17	1.30^{Ba}± 0.15

Higher concentrations of AC and BAP (2.5 g/L, 1.75 mg/L raised number of leaves (32.40±2.0 and 46.4±3.6) after 9 and 13 weeks respectively (Table 2). The minimum number of leaves was observed when there was no AC but the highest amount of BAP (1.75 mg/L) in the media. Even though increasing AC or BAP alone did not create a regular pattern on the number of leaves in any duration, after AC level was reached to 1.5 g/L, BAP concentration apperaed to be possitivley correlated with leaf number in week 13. As it has been reported previously, in our experiments, MS medium suplemented with activated charcoal also provided a better leaf growth compared to a medium without

 DOI: 10.18380/SZIE.COLUM.2017.4.1.suppl

activaed charcoal (Rittirat et al., 2012). Consisiting with results by Rittirat et al. (2012), in our experiment, MS medium suplemented with activated charcoal provided a better leaf growth compared to a medium without activaed charcoal. Higher number of leaves obtained by adding activated charcoal related to the attribution of AC in adsorption of inhibitory compounds in the culture medium (Fridborg et al., 1978).

Table 2. Number of leaves of F. imperialis plantlets.after 9, and 13 week. Means with different letters are significantly different at ($p<0.05$) as determined by Tukey's HSD test (Mean ± SD, n= 5).Capital letters represent AC effect, small letters represent BAP effect.

AC g/L	BAP mg/L	Number of leaves	
		9 weeks	13 weeks
AC_0	Cyt_0	1.60^{Aa}±0.5	8.0^{Ab}±1.2
	$Cyt_{1.5}$	1.40^{Aa}±0.5	2.2^{Aa}±0.8
	$Cyt_{1.75}$	1.20^{Aa}±0.4	1.6^{Aa}±0.5
$AC_{0.5}$	Cyt_0	6.60^{Ca}±1.1	15.2^{Bb}±2.3
	$Cyt_{1.5}$	5.60^{Ba}±0.5	10.0^{Ba}±0.7
	$Cyt_{1.75}$	6.80^{Ba}±1.3	13.2^{Cb}±1.3
$AC_{1.5}$	Cyt_0	3.00^{Aa}±0.7	16.6^{Bb}±5.6
	$Cyt_{1.5}$	10.0^{Cb}±2.1	19.6^{Db}±1.1
	$Cyt_{1.75}$	5.20^{Ba}±1.3	5.8^{Ba}±1.7
$AC_{2.5}$	Cyt_0	5.00^{Ba}±1.0	11.8^{Aa}±1.4
	$Cyt_{1.5}$	12.00^{Cb}±2.8	15.4^{Ca}±1.8
	$Cyt_{1.75}$	32.40^{Cc}±2.0	46.4^{Db}±3.6

Conclusions

Results of this experiment provide invulnerable evidence that activated charcoal can be added to the culture media for propagation of *F. imperials* by indirect organogenesis to improve the growth of regenerated plantlets, but excessive doses of activated charcoal should be avoided to prevent adverse results. Further studies are needed to standardize the propagation, since *F. imperialis* is slow in growth and culture medium faces unfavorable changes in pH and nutrient depletion.

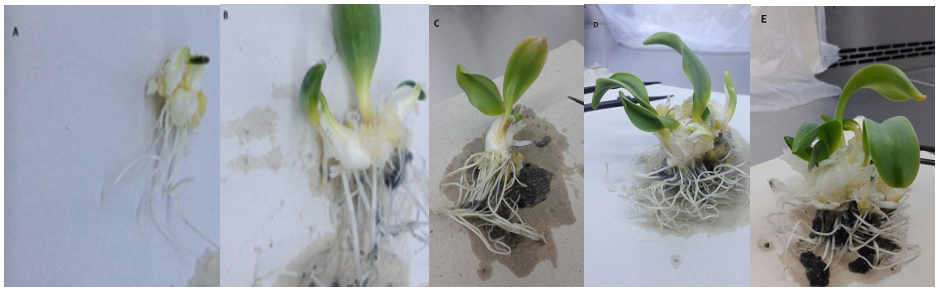


Figure 2. Plantlets of F. imperialis grown in different media: A- MS medium without AC, B- MS medium with 0.5 g/ L AC, C- MS medium with 1.5 g/ L AC, D- MS medium with 2.5 g/ L AC, E- MS medium with 1.5 g/ L AC and 1.5 mg/L BAP.

References

Almeida, R., Goncalves, S., Romano, A. (2005): In vitro micropropagation of endongered Rhododendron Ponticum L. subsp. Baeticum (Boissier and Reuter) Handel-Mazzetti. Biodiversity and Conservation. **14**:5.1059-1069. DOI: 10.1007/s10531-004-8413-3.

Bhatia, P., Bhatia, N.P., Ashwath, N. (2002): In vitro propagation of Stackhousiab tryonii Bailey (Stackhousiaceae): A rare and serpertine-endermic species of central Queensland, Australia Biodiversity and conservation .**11**:8.1469-1477. DOI: 10.1023/a:1016252207054.

Druart, Ph. and De Wulf, O. (1993): Activated charcoal catalyses sucrose hydrolysis during autoclaving. Plant Cell Tissue Organ Culture. **32**:1.96-99. DOI: 10.1007/bf00040122.

Dumas, E. and Monteuuis, O. (1995): In vitro rooting of micropropagated shoots from juvenile and mature Pinus pinaster explants: influence of activated charcoal. Plant Cell Tissue and Organ Culture. **40**:3.231-235. DOI: 10.1007/bf00048128.

Ebrahimie, E., Habashy, A.A., Mohammadie-Dehcheshmeh, M., Ghannadha, M.R., Ghareyazie, B, Yazdi-Amadi, B.(2006b): Direct shoot regeneration from mature embryo as a rapid and genotype-independent pathway in tissue culture of heterogeneous diverse sets of cumin (*Cuminum cyminum* L.) genotypes. In Vitro Cellular & Developmental Biology-Plant. **42**:5.455- 460. DOI: 10.1079/ivp2006789.

Fridborg, G. and Eriksson, T. (1975): Effects of Activated Charcoal on Growth and Morphologenesis in Cell Cultures. Physiologia Plantarum. **34**:4.306-308. DOI: 10.1111/j.1399-3054.1975.tb03843.x.

Fridborg, G. and Pedersen, M.L., Eriksson, T. (1978): The effect of activated charcoal on tissue culture: adsorption of metabolites inhibiting morphogenesis. Physiologia Plantarum. **43**:2.104-106. DOI: 10.1111/j.1399-3054.1978.tb01575.x.

Gao, S.L., Zhu, D.N., Cai, Z.H., Jiang, Y., Xu, D.R. (1999): Organ culture of a precious Chinese medicinal plant – *Fritillaria unibracteata*. Plant cell, tissue and organ culture. **59**:3.197-201. DOI: 10.1023/a:1006440801337.

Murashige, T. and Skoog, F. (1962): A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiologia Plantarum. **15**:3.473-497. DOI: 10.1111/j.1399-3054.1962.tb08052.x.

Nhut, D.T., Van, Le.B., Fukai, S., Tanaka, M., Van, K.(2001): Effects of activated charcoal, explant size, explant position and sucrose concentration on plant and shoot regeneration of *Lilium longiflorum* via young stem culture. Plant Growth Regular. **33**:1.59-65. DOI: 10.1023/a:1010701024963.

Pan, M.J. and Staden, J. (1998): The use of charcoal in in vitro culture-A review. Plant Growth Regulation. **26**:3.155-163. DOI: 10.1023/a:1006119015972.

Perry, L.M. and Metzger, J. (1980): Medicinal plants of East and Southeast Asia: attributed properties. Massachusetts institute of Technology Press, Cambridge, Massachusetts. **34**:4.361-361. DOI: 10.1007/bf02858311.

Rahimi, M., Daneshvar, M.H., Heidari, M. (2014): Propagation and bulb formation of Fritillaria (*Fritillaria imperialis* L.) via in vitro culture. Internation journal of Agronomy and Plant Production. **4**:3.707-710.

Rittirat, S., Thammasiri, K., Te-chato, S. (2012): Effect of media and sucrose concentration with or without activated charcoal on the plantlet growth of *P. cornu-cervi* (Breda) Blume& Rchb.f. Journal of Agriculture Technology. **8**:7.2077-2087.

Thomas, T.D. (2008): The role of activated charcoal in plant tissue culture. Biotechnology Advances. **26**:6.618-631. DOI: 10.1016/j.biotechadv.2008.08.003.

Wang, S., Gao, W., Chen, H., Xiao, P. (2005): New starches from *Fritillaria* species medicinal plants. Carbohydrate polymers. **61**:1.111-114. DOI: 10.1016/j.carbpol.2005.03.017.

Winkelmann, T. and Serek, M. (2005): Genotypic differences in callus formation and regeneration of somatic embryos in *Cyclamen persicum* Mill. Euphytica. **144**:1-2.109-117. DOI: 10.1007/s10681-005-5038-x.

 DOI: 10.18380/SZIE.COLUM.2017.4.1.suppl