KSÜ Tarım ve Doğa Derg 23 (2): 297-303, 2020
KSU J. Agric Nat 23 (2): 297-303, 2020
DOI:10.18016/ksutarimdoga.vi.627056

Effects of 6-benzylaminopurine and Activated Carbon on Indirect Organogenesis of Fritillaria Imperialis

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ABSTRACT

Fritillaria imperialis needs 2-3 years to regenerate from seeds, which makes its production quite challenging. In this study, effects of three concentrations of 6-Benzylaminopurine (BAP) (0.00, 0.10 and 0.15 mg/L) and three different amounts (0.0, 3.0 and 3.5 g/L) of activated carbon (AC) on the growth of *F. imperialis* plantlets *in vitro* were determined. The auxins were at similar concentration (0.01 mg/L of NAA and 0.01 mg/L of IAA) for all treatments. It has been found that the size and number of bulblets, as well as root and shoot numbers in regenerated plantlets were increased significantly ($p<0.05$) by adding 3.5 g/L of AC in culture medium supplemented with 0.10 mg/L of BAP. Moreover, addition of AC significantly reduced the length of roots in all doses studied ($p<0.05$). The supreme concentration of BAP (0.15 mg/L) decreased the bulblet number in all treatments, however, when supplemented with 3.5 g/L of AC, it was found to raise all the values measured ($p<0.05$). Overall, the best growth of the plant parts from callus were achieved with the highest concentration of AC and 1.5 mg/L of BAP.

Research Article

Article History
Received : 30.09.2019
Accepted : 11.11.2019

Keywords
Activated charcoal
Bulblet
Crown imperial
Cytokinin
Micropropagation

6-benzilaminopurin ve Aktif Karbonun Fritillaria Imperialis'in Dolaylı Organogenezi Üzerindeki Etkileri

ÖZET

Fritillaria imperialis türü bitkiler, tohumlarını yenilemek için iki yıldan fazla bir süreye ihtiyacı duymakta ve bu durum üretimlerini zorlaştırmaktadır. Bu çalışmada, üç farklı derişimdeki 6-Benzilaminopurinin (BAP) (0.00, 0.10 ve 0.15 mg/L) ve üç farklı miktardaki (0.0, 3.0 ve 3.5 g/L) aktif karbonun (AC) *F. imperialis* bitkilerinin büyümesine etkisi *in vitro* olarak incelenmiştir. Tüm muamele grupları için benzer konsantrasyonda oksin (0.01 mg/L NAA ve 0.01 mg/L IAA) kullanılmıştır. 0.10 mg/L BAP ilave edilmiş kültür ortamlarına 3.5 g/L AC eklenmesinin bitkilerin yumru çap uzunluğu ve sayısının yanında kök ve sürgün sayılarını istatiksel olarak anlamlı bir şekilde ($p<0.05$) arttırdığı gözlenmiştir. Dahası, AC eklenmesi, çalışılan tüm dozlarda kök uzunluklarını önemli ölçüde azaltmıştır ($p<0.05$). Yüksek BAP konsantrasyonunun (0.15 mg/L) çalışılan tüm grupların yumru sayılarını azalttığı, ancak 3.5 g/L AC ile desteklendiğinde ölçülen tüm değerlerin artışını sağladığı bulunmuştur ($p<0.05$). Genel olarak, kallustan bitki kısımlarının en iyi şekilde büyümesi, en yüksek konsantrasyonda AC ve 1.5 mg/L BAP ile elde edilmiştir.

Araştırma Makalesi

Makale Tarihçesi
Geliş Tarihi : 30.09.2019
Kabul Tarihi : 11.11.2019

Anahtar Kelimeler
Aktif karbon
Yumru
Ters lâle
Sitokinin
Mikropropagasyon

To Cite : Saeed N, Cömertpay S 2020. Effects of 6-benzylaminopurine and activated carbon on indirect organogenesis of *Fritillaria imperialis*. KSU J. Agric Nat 23 (2): 297-303. DOI: 10.18016/ksutarimdoga.vi.627056

INTRODUCTION

Fritillaria imperialis L. belongs to Liliaceae family, and. it can be used as ornamental plant and in flower arrangements. Besides, it is considered as a valuable plant material for medicine because of possessing steroidal alkaloids with multiple pharmaceutical properties (Eshaghi et al., 2018). The fact that the bulbs of *F. imperialis* have extreme amounts of

alkaloids and non-alkaloids led to their role in traditional drug in Turkey, China and Japan (Li et al., 2000; Wang et al., 2005). In addition to these remedies, most species of *Fritillaria* have high starch content and they can be used as new starch sources for the food production (Wang et al., 2005; Li et al., 2011).

Fritillaria imperialis usually lives more than two years, and the species belonging to *Fritillaria* genus are dispersed around different regions on the earth including Middle East (Iraq, Turkey, and Iran), Anatolia, Southern Asia and some places of United States (Rønsted et al., 2005). In the natural environment, a complete growth of the requires more than two years through sexual production (Sun and Wang, 1991). Moreover, the low rate of propagation from seeds hampers large scale cultivation of the species. Consequently, the number of bulbs produced in wild becomes insufficient to meet the demand adequately, therefore it is necessary to solve the problem by using *in vitro* techniques such as micropropagation (Almeida et al., 2005).

In vitro culture methods are essential components of plant genetic resources management and they are becoming more important for the conservation of endangered and rare plant species (Bhatia et al., 2002; Almeida et al., 2005). Micropropagation techniques can allow fast and large-scale propagation of plants. Regeneration without crossing callus is named as direct organogenesis. Indirect organogenesis, which includes the crossing of callus, has been applied for overcoming geophyte dependency and keeping long period to totipotency in *Cuminum cyminum* (Ebrahimie et al., 2006).

Under *in vitro* culture conditions, the growth of the bulblets of *F. imperialis* can be stimulated by a combination of plant growth hormones (Li et al., 2011). Suitable bulblet size for acclimatization is also achieved by transferring the plantlets to a growth medium containing no plant growth regulator (PGR) but an elevated level of carbohydrate contained with activated carbon (Staikkidou et al., 2000).

In *in vitro* culture, AC is widely used to promote the rooting of micropropagated plantlets since it helps the uptake of vitamins, ferritin, hormones, and darkens the medium (Dumas and Monteuuis, 1995). Besides, the detrimental substances generated by the explant or culture medium are adsorbed by AC (Fridborg and Eriksson, 1975; Fridborg et al., 1978). Cytokinin hormone is an important component of the medium for micropropagation, particularly at the proliferation stage. It has been widely known that cytokinin plays various roles in plant growth such as protein synthesis, simulation of division and cell expansion (George et al., 2008). This study investigates the impact of varying amounts of AC alone and combined with different concentrations of BAP. on the development of micropropagated *F. imperialis* grown in the media containing the same amount of Naphthaleneacetic acid (NAA) and Indole-3-acetic acid (IAA).

MATERIALS and METHODS

Plant materials

The plant materials were kindly provided by Prof. Dr. Mehmet Nuri Nas of Horticulture Department of Kahramanmaras Sutcu Imam University. The fresh bulbs before sterilization are seen in Figure 1. Soil particles, dead parts and insects were removed from the bulb surface by washing them thoroughly by tap water and rinsing with sterile water three times (Figure 2)

Figure 1. The fresh bulbs of *F. imperialis* before sterilization process

Şekil 1. Sterilzasyon işleminden önce F. imperialis'in taze soğancığı

Figure 2 Washing the samples with water

Şekil 2. Örneklerin su ile yıkanması

Growth medium

In our experiment, we used Murashige and Skoog (MS) basal medium to grow the explants. The medium pH was set to 5.5±0.9 and it was autoclaved at 121°C for 15 minutes before adding 5.5 g/L of agar plant (Duchefa Biochemie, Netherland) into the medium in order to provide it with a gel-like texture. As the energy and carbon source, 30 g/L of sucrose was added to the

medium. The plant materials were cultured in a growth room with a constant 25±2°C temperature and 66±4% humidity and 16/8 hours light/dark period (sustained by enlightening with white fluorescence).

Callus Formation

The clean bulbs were cut into smaller pieces and soaked in 1% (w/v) NaClO (sodium hypochlorite) (Hypo, Turkey) with Tween-20 (1%) (OGH, Germany) for fifteen minutes (Figure 3). Then, the surface-sterilized pieces were prepared washed extensively with distilled water for four times and cultured (Figure 4) on MS supplemented with 0.125 mg/L of TDZ (Thidiazuron) (Sigma-Aldrich, Germany).

Figure 3. The bulbs of *Fritillaria imperialis* in sterilization process.

Şekil 3. Fritillaria imperialis soğancıklarının sterilizasyonu

Figure 4 Surface-sterilization of the pieces before placing them on media

Şekil 4. Besiyeri içerisine koymadan önce yüzey sterilizasyonu

Experimental Set Up

Once the callus was generated, the parts with 2*2 cm^2 surface area were selected, and cultured in MS containing various amounts (0.0, 3.0, 3.5 g/L) of AC (Merck KGaA, Germany), three concentrations (0.00, 0.10, 0.15 mg/L) of BAP (Duchefa Biochemie, Netherland) and an equal concentration of auxins (0.01 mg/L NAA and 0.01 mg/L IAA). All the above-mentioned processes were performed under sterilized conditions.

Statistical analysis

IBM SPSS Statistics software was used to perform statistical analyses. In order to distinguish the effect of activated charcoal and hormones, and to find out their interaction, variance analyses were made by Two Way ANOVA. Groups with a p value lower than 0.05 were considered as significantly different ($p<0.05$). For the purpose of confirmation of the results of Two-Way ANOVA and to label the experimental and control groups depending on their similarities, Tukey's HSD post hoc test was applied.

RESULTS and DISCUSSION

We evaluated the impact of three different doses (0.0, 3.0 and 3.5 g/L) of AC by itself or in combination with three different amounts (0.00, 0.10, 0.15 mg/L) of BAP on the growth of the plantlets of *F. imperialis*, cultured via indirect organogenesis. When we made the first measurements on the 9th week, we found that the moderate amount (0.10 mg/L) of BAP along with the maximum dose of AC (3.5 g/L) produced more bulblets, roots and shoots (13.00±3.1, 23.40±5.6 and 16.60±1.9) compared to the control group (1.20±0.4; 14.60±2.0; 14.20±3.30) to which no AC and no BAP was applied (Table 1). When the plantlets were grown in the media containing no AC but with moderate concentration (0.10 mg/L) of BAP, the lowest number of roots and shoots (6.80±1.9 and 8.20±1.9) were generated. Our results showed that adding AC to the growth media stimulated and fed plants tissue more effectively and that helped the explants in modification and differentiation. These observations were in agreement with the reports authored by Sipayung and co-workers in 2018. Similarly, Olah (2017) demonstrated that AC could improve bulblet growth by possibly prohibiting exudates that cause tissue browning. Moreover, according to Cheng et al. (2013), AC could act as a promoter of embryogenesis in the microspore culture of different *Capsicum annuum* L genotypes.

After 13 weeks of treatment, number of roots, shoots and the bulblets were determined (Table 2). We found that adding AC to the media with no cytokinin has an increasing impact on the number of bulblets, roots and shoots in a dose-dependent manner, which reached their maximum level with the highest doses of AC as 8.40±1.8, 73.20±5.8, and 25.40+4.9, respectively. Besides, our results revealed that, of all conditions studied for 13 weeks, the medium containing the highest dose (3.5 g/L) of AC with 0.10 mg/L of BAP generated the greatest number (13.00±3.1, 76.60±17.7 and 32.00±7.5) of bulblets, roots and shoots respectively (Table 2).

Table1. Bulblet, Root and Shoot Number of *F. imperialis* plantlets after 9 Weeks of Growth.
Tablo 1. 9 Hafta Büyümeden sonra F. imperialis Bitkiciklerindeki Soğancık, Kök ve Sürgün Sayısı

AC g/L	BAP mg/L	Number of bulblets *Soğancık sayısı*	Number of roots *Kök sayısı*	Number of shoots *Sürgün sayısı*
0.0AC	0.00Cyto	1.20^{Aa}±0.4	14.6^{Ab}±2.0	14.20^{Ab}±3.3
	0.10Cyto	2.60^{Ab}±0.5	6.80^{Aa}±1.9	8.20^{Aa}±1.9
	0.15Cyto	1.80^{Aa}±0.4	8.40^{Aa}±1.5	10.20^{Aa}±1.7
3.0AC	0.00Cyto	3.40^{Aa}±0.5	16.80^{Aa}±3.6	12.80^{Aa}±1.9
	0.10Cyto	3.20^{Aa}±0.8	23.40^{Bb}±5.6	17.80^{Ba}±3.7
	0.15Cyto	2.40^{Aa}±0.5	24.00^{Bb}±4.7	16.60^{Ba}±3.8
3.5AC	0.00Cyto	5.20^{Aa}±1.3	15.80^{Aa}±2.4	17.20^{Ab}±4.3
	0.10Cyto	13.00^{Bb}±3.1	27.40^{Bb}±3.5	16.60^{Bb}±1.9
	0.15Cyto	8.20^{Ba}±1.9	11.00^{Aa}±1.8	11.20^{Aa}±1.3

Means within a column followed by same letters are not significantly different at (P<0.05) as determined by Tukey's HSD test (n=5). Capital letters indicate AC effect, small letters indicate BAP effect.

Table 2. Number of Bulblets, Roots and Shoots of *F. imperialis* after 13 Weeks of Growth.
Tablo 2. 13 Hafta Büyümeden sonra F. imperialis Bitkiciklerindeki Soğancık, Kök ve Sürgün Sayısı

AC g/L	BAP mg/L	Number of bulblets *Soğancık sayısı*	Number of roots *Kök sayısı*	Number of shoots *Sürgün sayısı*
0.0AC	0.00Cyto	2.20^{Aa}±0.4	31.80^{Ab}±5.8	12.20^{Aa}±1.9
	0.10Cyto	3.80^{Ab}±0.8	23.00^{Aa}±4.6	12.40^{Aa}±2.3
	0.15Cyto	4.40^{Ab}±0.5	19.00^{Aa}±3.8	18.60^{Ab}±3.2
3.0AC	0.00Cyto	5.20^{Ba}±1.3	43.00^{Ba}±1.2	23.00^{Ba}±2.9
	0.10Cyto	4.80^{Ab}±1.0	27.00^{Aa}±2.2	16.60^{Aa}±3.3
	0.15Cyto	8.80^{Bb}±2.1	48.00^{Bb}±8.6	20.60^{Aa}±4.2
3.5AC	0.00Cyto	8.40^{Ca}±1.8	73.20^{Cc}±5.8	25.40^{Bb}±4.9
	0.10Cyto	13.00^{Bb}±3.1	76.60^{Bc}±17.7	32.00^{Bb}±7.5
	0.15Cyto	8.20^{Ba}±1.9	59.20^{Cb}±3.7	22.40^{Aa}±2.8

Means within a column followed by same letters are not significantly different at P<0.05 as determined by Tukey's HSD test (n=5). Capital letters indicate AC effect, small letters indicate BAP effect.

Despite the fact that they worked on another species (*Narcissus tazetta*), Steinits and Yahel (1982) also showed that AC promoted the growth of bulblets, which was in line with our findings. The presence of AC in the growth media was likely to increase the efficiency of cytokinin hormone which in turn improves the growth of the explants (Nhut et al., 2001). Besides, activated charcoal was also known to remove the effect of inhibitory compounds from the medium (Anagnostakis, 1974). Expectedly, we have observed the lowest number of shoots and bulblets (2.20±0.4 and 12±1.9) in the culture medium containing neither AC nor BAP (Table 1). As for the roots, the fewest numbers (19.00±3.8) were determined in the medium containing the highest concentration of BAP (0.15mg/L) without AC (Table 2).

The inhibition observed in embryogenesis and morphogenesis of the plants grown in no AC containing media is most likely to be related to the presence of phenolic compounds in the growth medium, as it was previously reported by Nayanakantha et al. (2010) Although the concentration of BAP was more than ten folds less compared to our previous (Saeed and Cömertpay, 2017) work (1.75 mg/L), but the negative impact of the phenolic compounds was observed on root numbers in the absence of AC in the growth medium.

When the measurements obtained for both the 9th and the 13th week were compared, it was revealed the best growth performance of bulblets (number and diameter) were determined when moderate hormone concentration (0.10 mg/L) BAP and the highest dose of AC (3.5 g/L) were added to the media (Figure 5). These results were in accordance with of Staikkidou and coworkers (2000), who reported that the bulblets were grown better when PGR levels were reduced during the multiplication phase, which counts for the role of activated charcoal in adsorbing PGR. The highest amount (3.5g/L) of AC and moderate concentration (0.10 mg/L) of BAP increased the diameter of bulblets to 0.46±0.1 and 0.75±0.1 after 9 and 13 weeks of growth respectively (Figure 5).

Within 9 weeks of growth, the longest roots (3.50±0.5) were produced when the plantlets were treated with 0.10 mg/L of BAP accompanied by 3.5g/L of AC. Surprisingly, when the treatment duration was extended to 13 weeks, the plantlets grown in the media supplemented with 0.15 mg/L of BAP and a higher dose of AC (3.5 g/L) generated the longest root (10.00±1.5) (Figure 6).

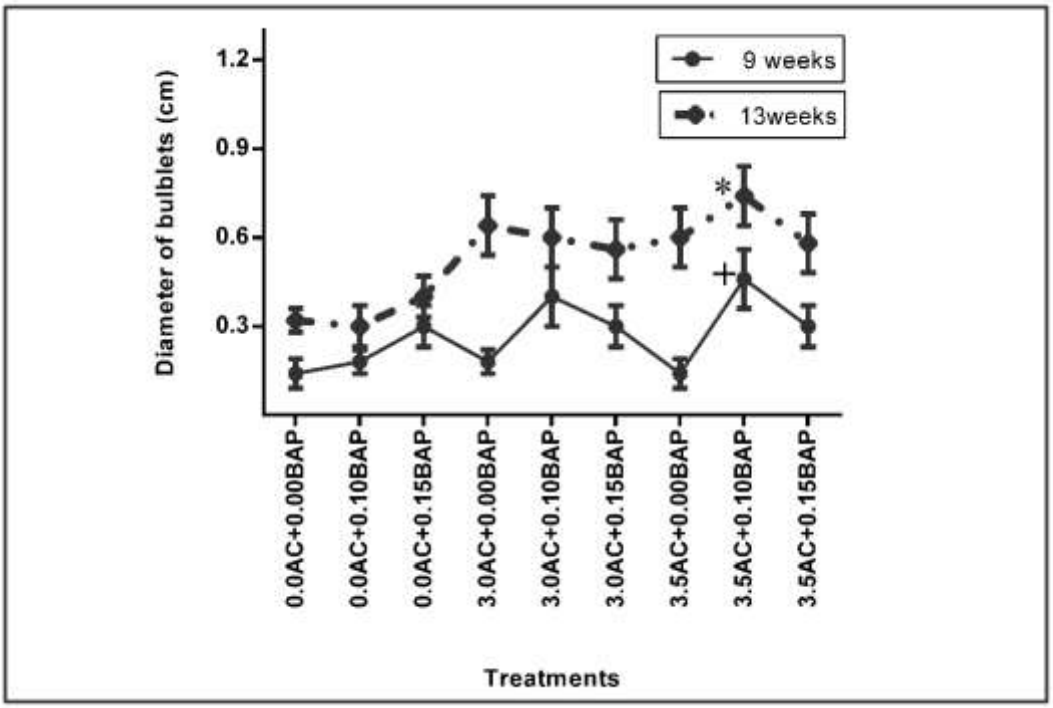

Figure 5. Bulblets Diameter of *F. imperialis* Plantlets after 9 and 13 weeks of Growth (n=5), (+): represents the best condition for bulblets diameter in 9 weeks. (*): represents the best condition for bulblets diameter in 13 weeks.

Şekil 5. 9 ve 13 haftalık büyümelerden sonra F. imperialis bitkiciklerinin soğancık çapları (n=5), (+) 9 haftalık büyüme için soğancık çapı bakımından en iyi koşulu temsil etmektedir. (*) 13 haftalık büyümeda soğancık çapı bakımından en iyi koşulu temsil eder

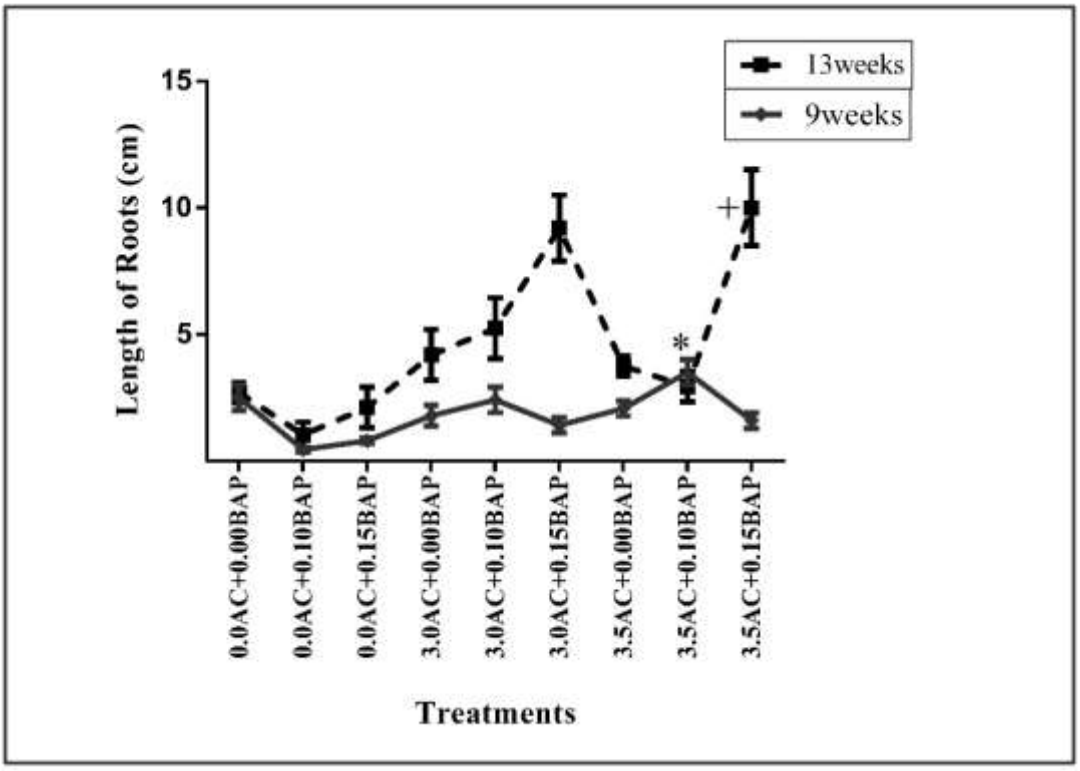

Figure 6. Root Length in Centimeter of *F. imperialis* Plantlets after 9 and 13 weeks (n=5), (*) represents the best condition for length of root in 9 weeks. (+) represents the best condition for length of root in 13weeks.

Şekil 6. 9 ve 13 haftalık büyümelerden sonra F. imperialis bitkiciklerinin santimetre cinsinden kök boyları (n=5), (*) 9 haftalık büyüme için kök boyu bakımından en iyi koşulu temsil etmektedir. (+) 13 haftalık büyüme için en iyi kök uzunluğu koşulunu temsil eder.

On the contrary, and regardless to the duration of the treatment, shorter roots were produced when 0.10 mg/L of BAP was used in the growth media with no AC. Similar to what we have observed, Staikkidou et al. (2000) stated that charcoal effected the development of the plantlets by inducing the formation of large number of roots and stimulating root elongation.

Some representative figures of plantlet development are presented in Figure 7.

CONCLUSION

Results of our study indicate the positive role of AC in the growth media on the development of the *F. imperialis* plantlets, especially on bulblet, root and shoot formation. The positive effects of AC is more pronounced at low concentrations of BAP with an adverse tendency at higher BAP levels. More studies are needed to address the interaction between nutrients and the AC in the growth media to standardize the application of the active carbon.

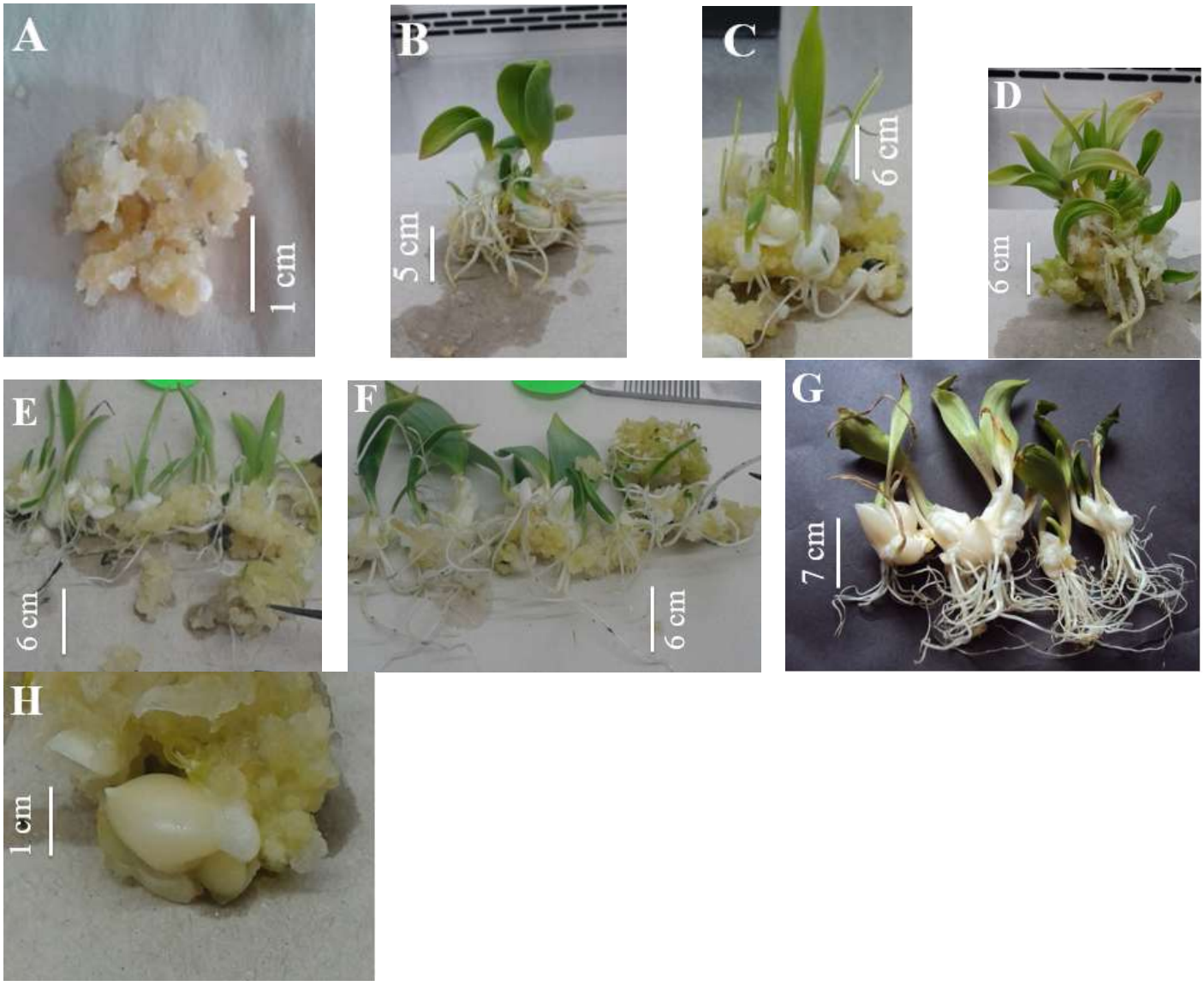

Figure 7. Plantlets of F. imperialis grown in different media
Şekil 7. Farklı besiyerinde büyümüş F. imperialis bitkicikleri.
(A)-Developed Callus on MS Medium after 40 Days of Incubation (*40 günlük inkübasyon sonunda MS besiyerinde gelişen kallus)*
(B)-MS Medium without BAP and AC (*BAP ve AC içermeyen MS besiyeri*)
(C) MS Medium with 0.10mg/ L BAP (*0.10mg/ L BAP içeren MS besiyeri*)
(D) MS Medium with 0.15 mg/ L BAP (*0.15 mg/ L BAP içeren MS besiyeri*)
(E) MS Medium with 3.0 g/ L AC (*3.0 g/ L AC içeren MS besiyeri*)
(F) MS Medium with 3.5 g/ L AC (*3.5 g/ L AC içeren MS besiyeri*)
(G) MS Medium with 0.10mg/L BAP and 3.5 g/L AC (*0.10mg/L BAP ve 3.5 g/L AC içeren MS besiyeri*)
(H) Regenerated Bulblet After 13 Weeks (*13 hafta sonunda üretilen soğancıklar*)

ACKNOWLEDGEMENTS

This work was supported by Scientific Research Projects (BAP) Unit of Kahramanmaras Sutcu Imam University with the project number of 2017/4-28D.

Statement of Conflict of Interest

Authors have declared no conflict of interest.

Author's Contributions

The contribution of the authors is equal.

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