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Development of an efficient callus production protocol for *Amsonia orientalis:* A critically endangered medicinal plant

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Abstract

Background: The plant blue star, rich in cardioactive and anti-cancer glycosides and glycoalkaloids and also used as an ornamental plant, is evaluated as "critically endangered" since it is nearly extinct in nature. In the present study, we describe a rapid and efficient callus production protocol for further studies that can be conducted on *Amsonia orientalis* such as secondary metabolite production and in vitro propagation.

Materials and Methods: Mature nodal explants were cultured on Murashige and Skoog media including 0.5 mg L^{-1} 6-benzylaminopurine. Obtained shoots were subcultured on same fresh media supplemented with different (0.5, 1.0, 2.0 or 4.0 mg L^{-1}) 6-benzylaminopurine concentrations. Explants from multiplied shoots were used for callusing experiments. Effects of various combinations of 6-benzylaminopurine, kinetin, indole-3-acetic acid and 2,4-dichlorophenoxyacetic acid on callus production were tested. At the end of the incubation period calli were weighted and means were compared using Duncan's multiple range test.

Results: When compared to others, all concentrations of 2,4-dichlorophenoxyacetic acid in combination with 6-benzylaminopurine were found to be the most effective on callus induction. Maximum mean callus weight of 0.327±0.07 g/callus was found at media supplemented with 0.5 mg L^{-1} 2,4-dichlorophenoxyacetic acid and 0.5 mg L^{-1} 6-benzylaminopurine in combination. Furthermore, higher kinetin concentrations caused production of fragile calli which are frequently used to initiate cell cultures.

Conclusions: The results have shown that media enriched with indole-3-acetic acid+kinetin combinations are more suitable for fragile calli production while media supplemented with 2,4-dichlorophenoxyacetic acid+6-benzylaminopurine combinations provide large but compact callus tissues. The findings will contribute to the establishment of cell suspension cultures.

Keywords: *Amsonia orientalis*, Apocynaceae, callogenesis, plant growth regulators, secondary metabolite production.

Abbreviations: 2,4-dichlorophenoxyacetic acid: 2,4-D; 6-benzylaminopurine: BAP; Critically endangered: CR; Indole-3-acetic acid: IAA; Kinetin: Kn; Murashige and Skoog: MS; Naphthaleneacetic acid: NAA; Plant Growth Regulator: PGR.

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INTRODUCTION

Amsonia Walter is a genus of Apocynaceae (dogbane) family. Members of this genus are erect perennial herbs, sometimes woody at base and also have latex like other members of dogbane family. *Amsonia orientalis* Decne. (syn. *Rhazya orientalis* (Decne.)) is a medicinal and ornamental plant which has very restricted natural distribution only in Turkey and North-East of Greece in the world (Tutin et al. 1968, Davis 1978). *A. orientalis*, according to the "Red Data Book of Turkish Plants", is shown in the category of "critically endangered" (CR) and as per the Bern Convention, placed by the European Council in the list of the plant species that must be conserved on European scale. *A. orientalis* is introduced into West European and American gardens as an ornamental plant for its profuse blue flowers, in cultivation it is more luxuriant, with looser inflorescences, than in the wild, where it is now very rare and near extinction (Davis 1978). According to Davis's Flora of Turkey, this species is distributed in Balıkesir, Bursa and İstanbul provinces but recent field studies have indicated that it is distributed only in Balıkesir and İstanbul but other populations are extinct (Özen 2006). The rare

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populations were found in Adnan Menderes, Gazi Osman Paşa and Paşaalanı districts of Balıkesir and Ömerli Dam area in İstanbul. These populations were taken under ex situ conservation at gardens in Umuttepe Campus of Kocaeli University in Kocaeli province of Turkey.

Unlikely to its relative *Nerium, Amsonia* sap doesn't have highly toxic alkaloids. Anatomy, morphology, palynology and antimicrobial activity of *A. orientalis* have been investigated. Antimicrobial activity tests of plant have shown that plant has a strong antimicrobial activity against several yeasts and bacteria (Akyalçın et al. 2006). *A. orientalis* is known to be a rich source of indole alkaloids. Furthermore, anticancer and anti-tumour activities of some of these alkaloids were declared by several researchers (Dabiné et al. 1986, Rahman and Zaman 1988, Rahman et al. 1989, Sauerwein 1991). A study about glycosidic constituents of *A. orientalis* was also carried out. Itoh et al. (2002) examined the constituents of the glycosidic fraction and succeed to isolate six new flavonoid glycosides. Propagation of *A. orientalis* through in vitro germination of seeds was carried out and callus formation was achieved by Öz et al. (2008).

Plant tissue cultures provide propagation of plants which are rare or economically important and can be used to induce quantitative and qualitative modifications on the production of plant secondary metabolites by changing nutrient and hormonal media culture conditions (Collin 2001). Both callus induction and plant regeneration from explants require the presence of appropriate concentrations and combinations of plant growth regulators in the culture media (Khan et al. 2011). Today, callus tissues and suspension cultures are used widely for production of plant secondary metabolites. The aim of this research was to develop efficient callus production protocol by testing the effects of culture media, supplemented with various concentrations of PGRs (Plant Growth Regulators) for the first time.

MATERIALS AND METHODS

Plant material

One of the mature plants taken from its natural habitat was selected as donor. Shoots taken from mature plant were cut into shorter pieces about 10 cm and washed under tap water for 15 min. Before the washing step, all leaves on shoots were cut off. Shoot pieces were disinfected by dipping in 70% ethyl alcohol for 2 min and 1% sodium hypochlorite for 12 min respectively. Disinfected shoots were placed in sterile distilled water and kept there for 5 min in order to take sodium hypochlorite residues away.

Shoot proliferation

Sterile shoots were cut into small segments which have a single node at least. Obtained sterile nodal segments were planted vertically in culture vessels containing 40 mL of Murashige and Skoog's (1962) basal media (MS) including vitamins and 0.5 mg L^{-1} 6-benzylaminopurine (BAP). At the end of the 4th w of incubation, the shoots derived from the buds of each explant were excised and their nodal segments were separated. Obtained nodal segments were subcultured on MS media supplemented with 0.5, 1.0, 2.0 or 4.0 mg L^{-1} BAP. At the end of the 4th w of incubation multiplied shoots were excised and used as explants for callusing experiments.

Culture media

Internodal explants from multiplied shoots were cut into 8-10 mm long pieces and horizontally planted onto MS media supplemented with varying levels (0.5-2.0 mg L^{-1}) of 2,4-dichlorophenoxyacetic acid (2,4-D)+BAP, indole-3-acetic acid (IAA)+kinetin (Kn) and IAA+BAP combinations. All media were supplemented with 30 g L^{-1} sucrose and 7 g L^{-1} of Plant agar (Duchefa). The pH of all media was adjusted to 5.7 with 1 N NaOH or 1 N HCl prior to autoclaving at 1.05 kg cm^{-2}, 121˚C for 20 min. Disinfection treatments and inoculations were carried out aseptically in a laminar air flow cabinet.

Culture conditions

Cultures were maintained at 23±1°C temperature in a plant growth chamber with a 16/8 h light/dark photoperiod under an illumination of 40 µmol m^{-2} s^{-1} photosynthetic photon flux intensity provided by cool-white fluorescent lights.

Data collection and statistical analysis

At the end of the 4th w of incubation calli were separated and weighed. Forms and colors of calli

were also noted. All experiments were repeated thrice and carried out with 15 replicates. Means were compared using Duncan's multiple range test at P≤0.05 significance level. The software IBM SPSS Statistics was used for statistical analysis.

RESULTS AND DISCUSSION

Shoot proliferation

The shoot proliferation rate from mature nodal explants was found to be 100% and no contamination was observed (Fig. 1a). Effects of BAP on the proliferation of in vitro derived shoots are presented (Table 1). The media supplemented with 0.5 mg L^{-1} induced maximum shoot proliferation rate (100%) while the highest number of shoots for per explant (3.76±0.92) was obtained from the media containing 1.0 mg L^{-1} BAP (Fig. 1b). The effects of media supplemented with 0.5 mg L^{-1} and 1.0 mg L^{-1} on the number of shoots for per explant were not significantly different. On the other hand, increase of BAP concentrations caused a decrease on mean shoot length. It can be considered that higher BAP concentrations caused reduction of shoot length due to the decrease of apical dominance.

Callogenesis

The effects of different concentrations of 2,4-D+BAP, IAA+Kn and IAA+BAP combinations on callus induction on MS media are presented (Table 2). Explant responses were obtained from wounded surfaces in 2 w. All concentrations of PGR combinations successfully induced callus formation at different percentages. When compared to others all concentrations of 2,4-D+BAP combination were found to be the most effective on callus induction. Maximum mean callus weight of 0.327±0.07 g/callus was found at media containing 0.5 mg L^{-1} 2,4-D in combination with 0.5 mg L^{-1} BAP while all other media containing IAA+BAP and IAA+Kn combinations were remarkably produced less. However, higher Kn concentrations caused production of fragile calli. Media with balanced hormonal concentrations had the highest percentage of callus production and callus fresh weight. It was expected that the concentration of studied auxins (2,4-D and IAA) stimulated callus initiation and growth. There were significant differences in callus formation depending on the type of auxin. In our study 2,4-D was more effective than IAA for callus formation. The effect of IAA on callus production was poor. This is in conformity with the data of Petrova et al. (2011). As it can be seen from Table 2, 2,4-D and BAP combinations could stimulate callus production well and both have showed synergy with each other during callus formation (Fig. 2a). Synergy between growth regulators on callus formation and plant regeneration was declared before several researchers (Tefera and Wannakrairoj 2006, Rani and Rana 2010, Chowdhury et al. 2011). When the cultures on media supplemented with 2,4-D+BAP combinations were kept on incubation after period of 4 w, they could neither induce shoots nor form roots. However, with increased culture time, the calli could develop continuously up to 8th w and later it turned into brown gradually. Shoot or root formation was observed at media supplemented with IAA+Kn and IAA+BAP combinations in time after 6th w. Especially on the media containing 2.0 mg L^{-1} IAA+0.5 mg L^{-1} BAP and 1.5 mg L^{-1} IAA+0.5 mg L^{-1} Kn calli formed roots finely within increased culture time (Fig. 2b). During the experiments, no fragile callus development was observed at media containing 2,4-D+BAP and IAA+BAP combinations. However, amongst others only 5 combinations of IAA+Kn (10.42% of all) gave fragile calli.

The seeds of *A. orientalis* were characterized by slow and uneven germination. Germination frequency of seeds was found to be around 15% by Öz et al. (2008). Culturing mature explants could be a more effective way for both in vitro propagation and callus production of *A. orientalis* than trying to germinate seeds for such purposes. 2,4-D is a growth regulator essentially needed for callus initiation and growth in most of the in vitro culture studies but it limits regeneration of plantlets (Sachiko and Hegazi 1990, Marshall and Diggle 2001). As have been reported, 2,4-D induce callus formation in a variety of species (Ma and Xu 2002, Thao et al. 2003). Findings of this research have revealed that 2,4-D has a key role in callus production of *A. orientalis* as well. After callus initiation, for regeneration

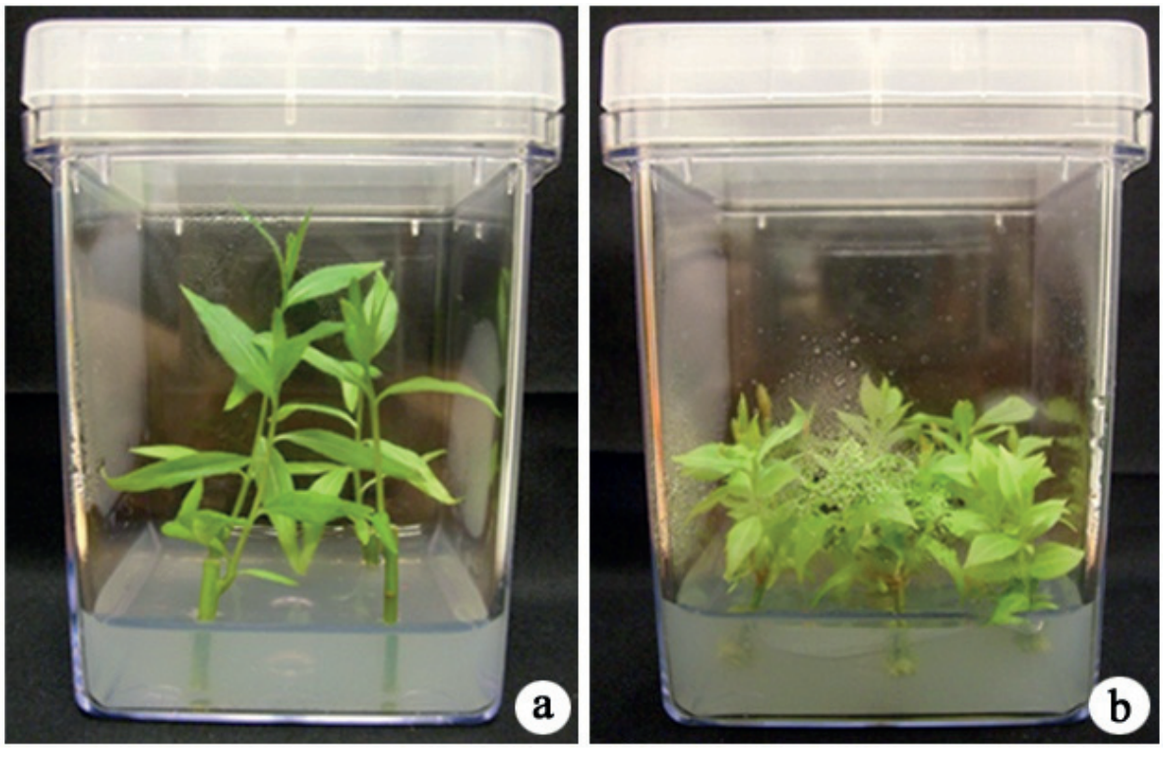


Fig. 1. Primary culture and subculture stages of experiment: a) Shoot proliferation from axillary buds of mature nodal segments on MS medium supplemented with 0.5 mg L^{-1} BAP; b) Subculturing phase on MS medium containing 1.0 mg L^{-1} BAP.

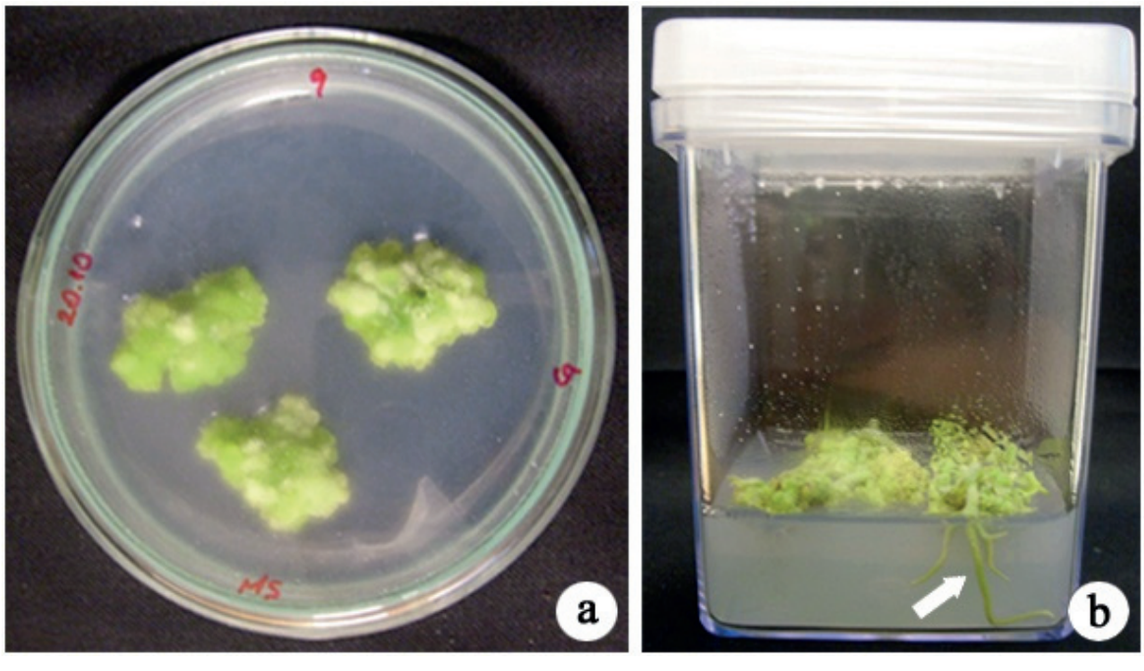


Fig. 2. Callus induction and root formation on callus cultures: a) Callus formation on MS medium containing 0.5 mg L^{-1} 2,4-D+0.5 mg L^{-1} BAP at the end of the 4th week of incubation; b) Root formation on callus tissue which cultured on MS medium containing 2.0 mg L^{-1} IAA+0.5 mg L^{-1} BAP after 7th w of incubation.

Table 1. Effects of BAP on the shoot proliferation of *A. orientalis*.

Concentrations of BAP (mg L^{-1})	Shoot proliferation (%)	No. of shoots/ explant	Mean shoot length(cm)
Control (MS)	82	1.38^{d}±0.49	1.15^{g}±0.47
0.5	100	3.60^{a}±0.84	3.06^{e}±0.63
1.0	91	3.76^{a}±0.92	2.18^{f}±0.60
2.0	76	3.24^{b}±0.70	1.31^{g}±0.44
4.0	71	2.69^{c}±0.78	0.82^{h}±0.32

Data represented average ± SD of three replicates, each with 15 explants.
Means having the same letter in a column were not significantly different by Duncan's multiple-range test (P≤0.05).

Table 2. Effects of different media on the callus production of *A. orientalis*.

Concentrations (mg L^{-1})	Growth regulator								
	2,4-D+BAP			IAA+Kn			IAA+BAP		
	Callus response (%)	Mean weight of callus (g)	Nature of callus*	Callus response (%)	Mean weight of callus (g)	Nature of callus*	Callus response (%)	Mean weight of callus (g)	Nature of callus*
0.5 + 0.5	100	0.327^{a}±0.07	C/Lg	93	0.047^{bcd}±0.03	C/Dg	93	0.064^{abc}±0.03	C/Lg
1.0 + 0.5	100	0.182^{cd}±0.09	C/Dg	93	0.026^{d}±0.02	C/Dg	53	0.021^{d}±0.01	C/Dg
1.5 + 0.5	100	0.232^{bcd}±0.08	C/Dg	87	0.027^{cd}±0.02	C/Dg	71	0.046^{bcd}±0.03	C/Dg
2.0 + 0.5	100	0.216^{bcd}±0.09	C/Dg	80	0.028^{cd}±0.02	F/Dg	73	0.068^{abc}±0.04	C/Dg
0.5 + 1.0	82	0.195^{bcd}±0.10	C/Dg	67	0.046^{bcd}±0.04	C/Dg	40	0.038^{cd}±0.03	C/Dg
1.0 + 1.0	100	0.256^{bc}±0.09	C/Dg	87	0.052^{abcd}±0.03	C/Dg	93	0.079^{a}±0.05	C/Dg
1.5 + 1.0	100	0.183^{cd}±0.10	C/Lg	82	0.058^{abcd}±0.03	C/Dg	100	0.075^{ab}±0.06	C/Dg
2.0 + 1.0	84	0.178^{d}±0.10	C/Dg	100	0.083^{a}±0.05	C/Dg	91	0.063^{abc}±0.03	C/Dg
0.5 + 1.5	91	0.263^{b}±0.08	C/Dg	33	0.067^{ab}±0.05	F/Dg	100	0.058^{abc}±0.04	C/Dg
1.0 + 1.5	78	0.184^{cd}±0.09	C/Dg	100	0.049^{bcd}±0.04	F/Dg	84	0.058^{abc}±0.05	C/Dg
1.5 + 1.5	80	0.218^{bcd}±0.10	C/Dg	100	0.061^{ab}±0.02	C/Lg	100	0.051^{abcd}±0.05	C/Lg
2.0 + 1.5	67	0.230^{bcd}±0.10	C/Dg	100	0.055^{abcd}±0.04	C/Dg	100	0.047^{abcd}±0.04	C/Dg
0.5 + 2.0	93	0.249^{bcd}±0.08	C/Dg	78	0.065^{ab}±0.05	F/Lg	76	0.021^{d}±0.01	C/Dg
1.0 + 2.0	80	0.215^{bcd}±0.08	C/Dg	87	0.059^{abc}±0.04	C/Lg	91	0.041^{cd}±0.03	C/Lg
1.5 + 2.0	80	0.187^{cd}±0.10	C/Dg	67	0.068^{ab}±0.02	C/Lg	80	0.069^{abc}±0.03	C/Dg
2.0 + 2.0	100	0.256^{bc}±0.07	C/Lg	87	0.062^{ab}±0.06	F/Lg	80	0.057^{abc}±0.04	C/Dg

Data represented average ± SD of three replicates, each with 15 explants.
Means having the same letter in a column were not significantly different by Duncan's multiple-range test (P≤0.05).
*C: Compact, F: Fragile, Dg: Green-Dark green, Lg: Green-Light green or whitish.

purposes callus should be transferred to a 2,4-D free media. Due to type of explant, amount of callus production can be different and internal hormone concentrations are decisive both for callus induction and regeneration. Although Öz et al. (2008) declared that they achieved callus induction in MS media containing 2.0 mg L^{-1} BAP plus 0.2 mg L^{-1} naphthaleneacetic acid (NAA) from shoot segments, our study showed that *A. orentalis* can easily produce callus at different percentages in any MS media.

Consequently, PGR combinations and concentrations significantly influenced the frequency of callus formation and shoot regeneration in *A. orientalis*. Callus cultures are originally used to produce cell suspension cultures to study the synthesis of secondary metabolites (Neumann et al. 2009). Additionally, essential oil accumulation only takes place in callus cultures. In order to optimize the

accumulation of secondary metabolites, callus cultures should be exposed to a range of concentrations of growth regulators such as BAP, zeatin, NAA and 2,4-D (Collin 2001). Loosely packed or friable callus is usually selected for initiating suspension cultures (George 2008). Friability is a pre-requisite for raising a fine cell suspension in liquid media. In vitro callus development strategy can be used as tool to protect the biodiversity and natural vegetation of *A. orientalis*. Furthermore, callus can be used for somatic embryogenesis or differentiations for regeneration of *A. orientalis* plant. In order to propagate the plant via indirect organogenesis and take a step to produce medicinally valuable alkaloids and glycosides, an efficient callus production protocol of *A. orientalis* has been described for the first time.

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Amsonia Orientalis İçin Etkin Bir Kallus Üretim Protokolünün Geliştirilmesi: Nesli Tükenmekte Olan Tıbbi Bir Bitki

Özet:

Giriş: Kardiyoaktif ve antikanser glikozitler ile glikoalkaloitlerce zengin olan, aynı zamanda süs bitkisi olarak da kullanılan mavi yıldız bitkisi, doğada neredeyse tükenmekte olduğu için "çok tehlikede" kategorisinde değerlendirilmektedir. Mevcut çalışmada, *Amsonia orientalis* üzerinde ileride yapılabilecek sekonder metabolit üretimi ve in vitro çoğaltımı gibi çalışmalar için hızlı ve etkili bir kallus üretim protokolü tanımlamaktayız.

Materyal ve Metot: Olgun nodal eksplantlar 0.5 mg L^{-1} 6-benzilaminopürin içeren Murashige ve Skoog ortamlarında kültüre alındı. Elde edilen sürgünler farklı (0,5, 1,0, 2,0 veya 4,0 mg L^{-1}) 6-benzilaminopürin konsantrasyonları ile desteklenmiş aynı ortamlarda altkültüre alındı. Çoğaltılan sürgünlerden elde edilen eksplantlar kallus oluşturma deneylerinde kullanıldı. Çeşitli konsantrasyonlardaki 6-benzilaminopürin, kinetin, indol-3-asetik asit ve 2,4-diklorofenoksiasetik asit'in kallus üretimi üzerine olan etkileri denendi. İnkübasyon dönemi sonunda kalluslar tartıldı ve ortalamalar Duncan'ın çoklu karşılaştırma testi kullanılarak karşılaştırıldı.

Bulgular: Diğerleriyle karşılaştırıldığında, 6-benzilaminopürin ile kombinasyondaki tüm 2,4-diklorofenoksiasetik asit konsantrasyonlarının kallus indüksiyonu üzerine en etkili oldukları bulundu. En yüksek ortalama kallus ağırlığı olan 0.327±0.07 g/kallus, 0.5 mg L^{-1} 2,4-diklorofenoksiasetik asit ve 0.5 mg L^{-1} 6-benzilaminopürin kombinasyonu ile desteklenen ortamlarda bulundu. Ayrıca, yüksek kinetin konsantrasyonları genelde hücre kültürlerini başlatmak için kullanılan kırılgan kallus oluşumuna neden oldu.

Sonuç: Sonuçlar göstermektedir ki, indol-3-asetik asit+kinetin kombinasyonları ile zenginleştirilmiş ortamlar kırılgan kallus üretimi için daha uygunken 2,4-diklorofenoksiasetik asit+6-benzilaminopürin kombinasyonları ile desteklenmiş ortamlar fazla fakat sıkı kallus dokuları sağlarlar. Elde edilen bulgular hücre süspansiyon kültürlerinin kurulmasına katkıda bulunacaktır.

Anahtar Kelimeler: *Amsonia orientalis*, Apocynaceae, kallogenesis, bitki büyüme düzenleyicileri, sekonder metabolit üretimi.