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Influences of basal media, growth regulators, explant type and photoperiod on callus competency and pigmentation of *Alkanna orientalis* L.

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Abstract

The goal of this research was in-vitro callus induction and red pigment production of Alkanna orientalis as a medicinal herb which belongs to Boraginaceae family containing valuable naphthoquinone derivates. The two different explants (leaf and leaf base) were subjected to abiotic factors such as different nutrient media (MS, B5 and M9), light and plant growth regulators (IAA and IBA). High frequency reproducible, prolific and compact calli formation was obtained from MS and B5 media supplemented with IAA, whereas high pigmentation was found in leaf base explants on M9 medium. Leaf base explant and dark conditions were found more effective for both callus formation and pigment production. The pigmentation at IBA was more than IAA, and the maximum level of pigmentation was observed on M9 medium with a combination of 1.0 mg/l IBA under dark. The factors that may be the most influential in the production of callus and red color pigment from A. orientalis have been determined. A. orientalis may be considered to be alternative plants for the A/S (alkannin/shikonin) production in vitro.

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1. Introduction

Boraginaceae family including some important *Alkanna, Arnebia, Echium, Lithospermum* and *Onosma* genus are well known as medicinal and dye plants containing valuable secondary metabolites such as hydroxynaphthoquinone pigments (alkannin, shikonin) and their derivatives [1]. The genus Alkanna with 50 species is an important member of the family [2]. Turkey is also one of the most important gene centres for the genus represented in the flora of Turkey by 41 taxa belonging to 36 species with an 80% endemism rate [3]. *Alkanna* species are spread out in Mediterranean coast, Central, Eastern and Western Anatolia of Turkey the genus naturally grows in rocky, sandy and steppe habitats [4]. The most important phytochemical constituents of Alkanna taxa consist of isohexenylnaphthazarins, mainly alkannin, shikonin and other derivatives such as acetylalkannin, propionylalkannin, isobutylalkannin, angelylalkannin, β,β-Dimethylacrylalkannin, isovalerylalkannin and others [5-7]. The species are ethnobotonically used for many therapeutic and non-therapeutic purposes in worldwide. Their roots of the species are multipurpose utilizated in industries as a natural source of red pigments, including dye of cosmetics, food and textiles [8, 9]. Moreover, they are evaluated for treatment of a wide range of disorders such as a natural remedy to prevent and to treat ulcers, wounds, various dermatological diseases, fever, inflammation, aging and herpes [6, 9, 10]. The scientific literature demonstrated the chemo-preventive [11], antiproliferative [8], anticancer [12], wound healing [13], antimicrobial [14], antipyretic, antinociceptive and sedative [15] properties of *Alkanna* species root bark.

The biotechnological approaches such as cell/tissue culture have been efficiently used as alternative renewable source for the industrially large-scale commercial production of these pharmaceutical compounds resembling those accumulated in the root bark of the original plants [16-19]. Many studies have

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reported that the bioactive compounds obtained from tissue culture techniques are minimal for production time and the cost of labor than those obtained from field-grown plants [20, 21]. Callus, suspension and root culture among tissue culture techniques are efficient approach to produce fast, stable and prolific secondary metabolites [22]. Callus cultures were successfully applied to produce secondary metabolites for many plant species. The induction of regenerative and compact callus is accomplished by differential application of various plant growth regulators (PGRs) types and control of conditions in the culture medium [23]. Different plant tissue culture approaches such as different basal media, combinations and concentrations of PGRs, explant types, have been used to increase the production of A/S and its derivatives [24] along with genetic manipulation [25]. Commercial production of A/S and its derivatives were first produced by callus culture of *Lithospermum erythrorhizon* [26]. It is also reported that A/S and it derivatives might be produced using tissue culture techniques for different species, e.g., callus culture of *Alkanna tinctoria* [27], suspension culture of *Alkanna orientalis* [17], callus culture and suspension culture of *Arnebia hispidissima* [28], suspension culture of *Echium italicum* L. [29], root and suspension culture of *Lithospermum canescens* [30], as well as callus culture of *Onosma bulbotrichom* [18].

Alkanna orientalis (L.) Boiss var. *orientalis* is a perennial deciduous herb with red-pigmented roots [31], and grows in wild regions of Mediterranean. Up to date, there are limited callus differentiation and shoot regeneration literatures about the production of *in vitro* secondary metabolities in *Alkanna* species [17, 32]. In our previous study, callus induction and regeneration capacity of *Alkanna orientalis* were achieved using different type and concentrations cytokine/auxin combination [32]. However, it is necessary to develop reliable and efficient methods to increase the frequency of induction of morphogenesis in calli. The present study also aimed to improve suitable and prolific callus culture system and red pigmentation in *Alkanna orientalis* using different nutrient basal media and growth regulators.

2. Materials and Methods

2.1. Material

The seeds of *Alkanna orientalis* (L.) Boiss var. orientalis were collected from Yozgat Bozok University Campus (1339 m, 39° 46′ 45″N, 34° 47′ 38″E) in June 2017, Yozgat province, in Turkey (Figure 1) dried at room temperature for 7 days and kept under 4 °C.

Figure 1. *Alkanna orientelis* at its natural habitat in Yozgat province of Turkey

2.2. Seed surface sterilization

Seed sterilization and inoculation were performed as described by Yaman et al. [32]. The seeds of *A. orientalis* were thoroughly washed in a running tap water for 30 min. Then, the seeds were surface sterlizated with 20% (v/v) commercial bleach (Axion) containg sodium hypochlorite (0.4-0.5%) for 20 min and rinsed three times with de-ionized sterile water. Subsequently, surface sterilized seeds were kept in sterile distilled water for 1 h to remove the hard and thick shell of seeds. The thick cortex of seeds was gently removed using sterile scalpel.

2.3. Germination and explant isolation

The surface sterilized seeds were germinated and cultured on MS [33] medium with vitamins supplemented with 0.25 mg/L BAP (6-benzylaminopurine), 0.5 mg/L Kn (kinetin), 1.0 mg/L IAA (indol acetic acid), 30 g/L sucrose and 6 g/l agar as previously described by Yaman et al. [32]. The pH was adjusted to 5.7-5.8 using 1 M NaOH. The plantlets of rosette plant growing from the germinated seeds were subcultured on the same medium after 15 days. The leaf and leaf base (the part where the leaf attaches to the stem) were excised from 4-week-old in vitro propagated seedlings. Leaf (2-3 mm width) and leaf base (1-2 mm length) of the species were cultured on MS, Gamborg B5 (B5) [34], and M9 [24] basic basal mediacontaining 30 g/l sucrose and 0.5, 0.1 and 2.0 mg/l IAA alone or indol butyric acid (IBA).

2.4. Culture conditions

The pH of all media was adjusted to 5.7-5.8 using 1 N NaOH followed by the addition of agar. The medium-containing flasks were then autoclaved at 121°C under 103 kPa for 20 min. Plant growth regulator (PGR)-free medium (MS0, B50 and M90) was used as the control.

All of the leaf and leaf base explants were separately inoculated onto these media. All cultures were separately incubated at 24 - 25 °C under 60% humidity. Each application was cultured in two different lighting conditions as follows: a 16/8 hrs photoperiod with 35 µmol m2/s light intensity delivered by cool white fluorescent (1) and darkness (2) conditions. Callus induction and pigmentation from explants in each application were recorded after 6 week from culture initiation. Also, the rosette plantlets developing from the germinated seeds of *A. orientalis* cultured separately MS, B5 and M9 mediums supplemented with 0.25 mg/L BAP, 0.5 mg/L Kn, 1.0 mg/L, 30 g/L sucrose under light. After 30 days, the observation of the seedlings growing on these three nutrient media was taken.

2.5. Experimental design and statistical analysis

Experiments were conducted in a completely randomized design and repeated three and each experiment consisted of 10 explants per Petri dishes (90 × 90 mm). The statistical comparison was conducted using version IBM SPSS statistics 20. Data given in percentage were subjected to arcsine (_X) transformation before statistical analysis. Duncan's multiple range tests was used to determine the significance at $P < 0.05$.

3. Results and Discussion

3.1. Effects of MS basal media on compact, prolific callus induction and pigmentation

The prolific callus induction and pigmentation from the leaf and leaf base explants of *Alkanna orientalis* were tested using different concentrations of IAA and and IBA on MS basal medium under light and darkness conditions.and all data were given in Table 1. Callus regeneration was significantly affected by different doses of IAA/IBA and darkness and light conditions and explant types. There were also statistically significant interactions between these parameters at $P < 0.05$.

Under darkness conditions, mean callus formation ratio (81.3%) was higher than the light application (34.6%). As the source of explant, the leaf base explant showed a higher callogenezis with 76.4% than the leaf explant (39.4%). Among auxin types, IAA was more effective on callus induction than IBA (42.5%), callus formation increased with increasing concentrations of IAA and IBA. Bagheri et al. [18] reported that callus initiation of *Onosma bolbutrichum* on MS media supplemented with increasing of IAA concentration in media increased and pigment production was observed on the entire surface of the callus tissue. In this study, the callus formed a brown color pigment. Leaf base explant exhibited 100% callus formation in all doses of IAA in darkness conditions and 2.0 mg/l IBA. 100 % callus regeneration was only achieved on the medium containing 2.0 mg/l IAA under light conditions. They also formed brownish-lack pigment, not red. Gharehmatrossian et al. [35] defined that callus of *Onosma sericeum* on MS media supplemented with 0.5 mg/1 IAA and 3.0 mg/l BAP under darkness were semi hard and black color.

Table 1. Effects of MS medium containing different doses of IAA and IBA on callus regeneration (%) of *A. orientalis* under light and darkness conditions

PGRs	Concentration (mg/l)	Light		Darkness		Mean	Mean
		Leaf	Leaf base	Leaf	Leaf base		
IAA	0	0.0b	0.0e	0.0c	0.0c		
	0.5	0.0b	53.3c	87.5a	100a	60.2b	
	1.0	40.0a	86.7b	93.8a	100a	80.6a	73.3
	2.0	25.0ab	100.0a	91.7a	100a	79.2a	
	Mean	21.7C	80.0B	91.0AB	100A		
IBA	0.5	0.0b	20.0d	4.2c	70.8b	23.8b	
	1.0	0.0b	0.0e	33.3b	95.8a	32.3b	42.5
	2.0	0.0b	90.0b	95.8a	100a	71.5a	
	Mean	0.0C	36.7B	44.4B	88.9A		
Mean		10.8C	58.3B	68.1B	94.4A		
Mean		34.6		81.3			
Leaf						39.4	
Leaf base						76.4	

The duncan comparisons of the difference between the data were showed with lower case letters in the line and upper case letters in the column

3.2. Effects of B5 basal media on compact, prolific callus induction and pigmentation

The responses of the leaf and leaf base of *A. orientalis* to different doses of IAA and IBA in B5 basal medium under light and darkness conditions were investigated and the data were given in Table 2.

As a result of the analysis, light/darkness conditions, explant source and auxin types showed different effects on callus induction. A higher rate of callus formation was observed in dark condition (66.0%) than light condition (36.8%). The leaf base explant produced a higher callus formation (80.8%) than the leaf explant (22.0%). B5 medium containing different concentrations of IAA produced higher mean callus ratio (60.1%) than B5 medium supplemented with various concentarations of IBA (42.7%). Leaf base explant exhibited 100% callus formation on BG medium including 0.5-1.0 mg/l of IAA and 1.0-2.0 mg/l IBA at darkness conditions, 0.5 mg/l IAA and 2.0 mg/l IBA under the light conditions. Leaf explant only gave 100% callus formation in 1.0 mg/l IAA under darkness conditions. The color of calli was brownish-lack pigment. Bagherieh-Najjar and Nezamdoos [36] stated same findings for pigment production from *Onosma dichroantha* in B5 medium. Similarly, Zare et al. [29] reported that the calli in B5 medium supplemented with 1 mg/L IAA and 1 mg/L kinetin failed to produce pigments and B5 medium appeaed to lack such potential which seems to be a key determinant factor for hydrophobic metabolites (naphthazarin derivatives etc.) that require a lipophilic adsorbent.

Electrostatic interactions between chemical species vary depending on the pH of the aqueous solution. In the extraction experiments, the interaction between the analyte and the selected chemical medium should be high.

Table 2. Effect of B5 medium containing different doses of IAA and IBA on callus regeneration (%) from leaf explant of *A. orientalis* under light/darkness conditions

PGRs	Concentration (mg/l)	Light		Darkness		Mean	Mean
		Leaf	Leaf base	Leaf	Leaf base		
IAA	0.0	0.0b	0.0d	0.0c	0.0d		
	0.5	0.0b	100a	0.0c	100a	50.0	
	1.0	0.0b	53.3bc	100a	100a	63.3	60.1
	2.0	26.7a	78.3b	75.0b	87.5b	66.9	
	Mean	8.9C	77.2AB	58.3B	95.8A		
IBA	0.5	0.0b	56.7bc	0c	66.7c	30.8b	
	1.0	0.0b	26.7c	0c	100a	31.7b	42.7
	2.0	0.0b	100a	62.5b	100a	65.6a	
	Mean	0.0C	61.1B	20.8C	88.9A		
Mean		4.5D	69.2B	20.8C	77.4A		
Mean		36.8		66.0			
Leaf						22.0	
Leaf base						80.8	

The duncan comparisons of the difference between the data were showed with lower case letters in the line and upper case letters in the column

3.2. Effects of M9 basal media on compact, prolific callus induction and pigmentation

The callus initiation and red pigment production from the leaf and leaf base of *A. orientalis* to different doses of IAA and IBA in M9 nutrient medium in light and darkness conditions were examined and the data were given in Table 3 and Table 4.

Table 3. Effect of M9 medium containing different doses of IAA and IBA on callus regeneration (%) from leaf explant of *A. orientalis* under light/darkness conditions

PGRs	Concentration (mg/l)	Light			Darkness		
		Callus rate	Nigrescence	Red pigment formation	Callus rate	Nigrescence	Red pigment formation *
IAA	0.0	0.0	+	0	0.0	+	0
	0.5	0.0	+	0	0.0	+	0
	1.0	0.0	+	0	0.0	+	0
	2.0	0.0	+	0	0.0	+	0
IBA	0.5	0.0	+	0	0.0	+	1
	1.0	0.0	+	0	0.0	+	1
	2.0	0.0	+	0	0.0	+	1

* 0, no red color. 1, 2, 3 and 4 denote the intensity of red color

As shown in Tables 3 and 4, virtually no callus was produced from both explants cultured at all dose concentrations of IAA and IBA in M9 medium light and dark conditions. Also, the leaf and leaf base explants cultured under light condition turned black in all nutrient medium. Zhang et al. [37] notified that white light completely blocked the biosynthesis of shikonin and its derivatives that produce the red pigment in the cells cultured in M9 medium.

Table 4. Effect of M9 medium containing different doses of IAA and IBA on callus regeneration (%) from leaf base explant of *A. orientalis*

PGRs	Concentration (mg/l)	Light			Darkness		
		Callus rate	Nigrescence	Red pigment formation	Callus rate	Nigrescence	Red pigment formation*
IAA	0.0	0.0	+	0	0.0	+	1
	0.5	0.0	+	0	0.0	+	1
	1.0	0.0	+	0	0.0	+	1
	2.0	0.0	+	0	0.0	+	1
IBA	0.5	0.0	+	0	0.0	+	2
	1.0	0.0	+	0	0.0	+	4
	2.0	0.0	+	0	0.0	+	3

* 0, no red color. 1, 2, 3 and 4 denote the intensity of red color

When the culture of both explants under dark conditions is examined, leaf explant formed a red pigment in all concentrations of IBA. But, all concentrations of IAA were not effective on pigment production from leaf explants. The leaf base explant generated red color pigment in all concentrations of IAA and IBA. Densities of color pigments were evaluated as 1, 2, 3 and 4 from less to more, respectively. While the color pigment density of leaf base explants in IAA growth regulator was at the level of 1 (Figure 2A), it was recorded that the leaf base explants cultured in all doses of IBA was formed the higher red pigment density (Table 4). The highest red pigment density, level 4, was determined in leaf base explants in 1.0 mg/l IBA under darkness conditions (Figure 2B). Among the various auxin types analyzed, many researcher indicated that IBA was more effective than IAA and NAA (a-naphthalene acetic acid) in inducing roots for *Arnebia hispidissima* containing shikonin in its root [38, 39]. Bagheri et al. [18] noted that IAA had lower callus induction than 2,4 D, whereas it was more effective to produce naphthoquinone pigments from callus of *Onosma bulbotrichom*, and, as the IAA concentration increased, the pigmentation in the calli decreased.

Figure 2. The red pigmentation from *A. orientelis* in M9 medium A) The red color pigment (level 1) in leaf base explants in IAA under darkness conditions B) The red color formation (level 4) from leaf base explants on M9 medium containing 1.0 mg/l IBA under darkness conditions after 4 weeks of culture initiation

In generally, the rosette plantlets developing from the germinated seeds of *A. orientalis* cultured separately MS, B5 and M9 media supplemented with 0.25 mg/L BAP, 0.5 mg/L Kn, 1.0 mg/L, 30 g/L sucrose under light. As indicated in Figure 3. M9 medium was found to be more effective on red pigment production of *A. orientalis* than MS and B5 media *in vitro* seedlings of *A. orientalis*. Previous studies clearly demonstrated that M9 medium had a higher level of red pigment production than MS, B5 and other media, in tissue culture of Boraginaceae species containing naphthazarin compounds in their roots [17, 36, 38].

Figure 3. *In vitro* seedlings of *A. orientalis* in different nutrient media under light conditions at the end of the 30 days of culture (A: MS, B: B5, C: M9)

Also, Fang et al. [40] recorded that no pigmentation was visible in hairy roots of *Lithospermum erythrorhizon* cultured in B5 medium under light condition, whereas distinct red color was apparent upon visual inspection when hairy roots were transferred into M9 medium in the dark conditions. The current work supports the studies developed by Fang [40] and Fujita [24] to produce industrially large quantities of important naphthazarine compounds (red pigment). For the production of red color pigments, primarily the cell proliferation culture of plant cells should be done on B5 nutrient medium, then these cells should be transferred to M9 liquid medium for red pigment production. In contrast to B5 medium, M9 medium lacks ammonium ions that are known to inhibit biosynthesis of naphthazarin and its derivatives [24]. Interestingly, although light signals mostly stimulate the biosynthesis of secondary metabolites [41], the red pigment production in analyzed all medium is completely inhibited under white light.

In our study the pigmentation was observed on nearly all explants in all nutrient media tested in the first week, however, the pigmentation density and color changed according to the nutrient medium, growth regulator and explant types in the following days. Similarly, Gupta et al. [42] reported that light had a negative effect on shikonin production in M9 medium in *Arnebia* sp., but even in light condition, shikonin production increased up to the first 4 days and inhibited in the following days, however, continued to increase in dark conditions.

Explant source is of great importance for plant regeneration and commercially secondary metabolite production of cell/organ through plant tissue culture. In species belonging to Boraginaceae, leaf and root explants are generally used to produce shikonin and its derivatives. The leaf base explants were higher regeneration capacity and pigmentation ability than the leaf in all tested applications. Yaman et al. [32] also recorded that both stem and leaf base explants induced high compact callus production (>86.0%) of *Alkanna orientalis* and *Alkanna sieheana* in all media under light.

Conclusion

In this study, the callus and red pigment production from leaf and leaf base *A. orientalis* in three basal nutrient media (MS, B5 and M9) supplemented with various concentrations of IAA or IBA under light and darkness conditions were examined. MS was the most suitable nutrient medium for both callus growth and brown pigment production (non-red). B5 medium was better for callus growth, but not well for pigment production. M9 medium was also found to be the best nutrient medium for red pigment production, but not a suitable nutrient medium for callus growth. In all media tested, leaf base explant showed higher regeneration capacity and pigmentation ability than leaf explant. Also, the findings of this study revealed that IAA had a powerful effect on callus induction,

while IBA was more effective on pigmentation. These data presented here are useful for large-scale *in vitro* micropropagation of *A. orientalis* for *in vitro* production of its valuable phytochemical compounds.

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Conflicts of interest

The authors state that did not have conflict of interests.

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