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THE EFFECT OF SOME ANTIBIOTIC AND FUNGUCIDE APPLICATIONS ON THE MICROPROPAGATION OF *Lilium candidum* L.

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Abstract

Lilium candidum is one of the important commercial crop worldwide. In this study, a protocol for micropropagation of Lilium candidum was developed the effect of some antibiotic and fungicide applications and also the effect of the light.
In experiments, bulb scales of Lilium candidum L. were used. The plant material was sterilized superficially and it was dipped in solutions which contain antibiotic and fungicide 30 minutes was cut into pieces and cultured in Murashige and Skoog (1962) medium supplemented with 0.1 mg/L NAA + 0.01 mg/L BA, 30 mg/L sucrose and 8 mg/L agar. The application of antibiotic and fungicide was the application of Streptomycin + Penicillin, Streptomycin + Benomyl and Benomyl + Nystatin in every two different doses.
Bulb scales were cutting into lateral as 5-10 mm pieces. The explants were cultured 16 hrs light - 8 hrs dark photoperiodic condition; the light was 1600 lux, and also were cultured in completely darkness condition in the same applications. Culture room temperature was between 19° and 22 °C.
Bulblet formation (%) was observed in photoperiodic condition higher than darkness one. In the Benomyl + Nystatin solution bulb formation (%) was the highest (97.4%). In addition the best application was obtained Benomyl + Nystatin solution both in respect of sterilization and the point of view the number of the bulblets.
Keywords: *Lilium candidum,* micropropagation, antibiotic, fungucide

Lilium candidum L.'nin MİKROÇOĞALTIMINDA BAZI ANTİBİYOTİK ve FUNGUSİT UYGULAMALARININ ETKİSİ

Öz

Bu çalışmada, dünya çapında ticari öneme sahip olan Lilium candidum L.'nin mikroçoğaltımında bazı antibiyotik ve fungusit uygulamalarının in vitro'da ki etkisi, uygun sterilizasyon ve ışığın etkisi araştırılmıştır.
Denemelerde temel MS (1962) ortamı kullanılmış, ortama 0.1 mg l/L NAA+ 0.01 mg l/ L BA, 30 g /L şeker ve 8 g /L agar ilavesi yapılmıştır. Bitki materyali (soğan pulları) yüzeysel sterilizasyonu takiben antibiyotik ve fungusit içeren solusyonlarda 30 dk bekletildikten sonra parçalara ayrılarak kültüre alınmıştır. Antibiyotik ve fungusit uygulamaları: iki farklı dozda Streptomycin + Penicilin uygulamaları, iki farklı dozda Streptomycin + Benomyl uygulamaları ve iki farklı dozda Benomyl + Nystatin uygulamalarıdır.
Lilium candidum soğan pulları 5-10 mm boyutunda enine parçalara ayrılarak kültüre alınmıştır. Karanlık ve fotoperiyodik koşulda (16 saat aydınlık- 8 saat karanlık) kültüre alınan eksplantlarda % soğancık oluşumunun, fotoperiyodik koşulda karanlığa göre daha yüksek olduğu gözlenmiştir. Ayrıca yüzeysel sterilizasyondan sonra yapılan uygulamalarda hem sterilizasyon yönünden hem de eksplant başına elde edilen soğancık sayısı bakımından en olumlu uygulamanın Benomyl + Nystatin solusyonlarının olduğu belirlenmiştir.
Anahtar Kelimeler: *Lilium candidum,* mikroçoğaltım, antibiyotik, fungusid

1 Introduction

Plant tissue culture have many usage areas. Among these, micropropagation occupies an important role as an alternative to the traditional plant reproduction techniques. Micropropagation with *in vitro* techniques which interests very much both studying practically and understanding physiological and biochemical studying occupies a popular research subject especially producing gardening and ornamental plants. Although increasing with tissue culture is an expensive techniques because of advantages it supplies nowadays it is used to produce many plants commercially [1,2]. All over the world one of the leading cut flowers and important ornemantal plants is *Lilium* [3,4]. *Lilium candidum* that is an aromatic and ornamental plant is a rare plant and grows in south west Anatolia, Turkey [5]. It is an endangered plant because of excessive collection from the wild [6]. In our country, *Lilium candidum* produced by bulb scales, bulblets and bulbils especially grow in Mediterrean regions. There are a lot of studies which have been investigated about *Lilium* species propagated from scale by micropropagation and also *in vitro* regeneration from scales of *Lilium* [7-11]. These studies associated with plant growth regulators -such as auxin and cytokinin concentrations- [3, 4, 7, 12-16], light application [11, 17-20], scale position and explant size [7, 12, 17, 21, 22], sucrose concentration [7, 11, 12, 22-24]. There are no studies on micropropagation of *L. candidum* about effect of some antibiotic and fungicide applications. In this study, our aims were to develop a successful

micropropagation protocol through tissue culture as an alternative other producing methods. Therefore, in this study bulb scales of *L. candidum* were used as an explant under suitable MS medium under photoperiodic and dark conditions using 5- 10 mm size explant antibiotic and fungicide applications were performed and its micropropagation were succeeded.

2 Material and Methods

Collection of explants: The bulbs of *Lilium candidum* were collected at the end of May from Dalyan- Muğla (in Turkey). The bulb scales of *L. candidum* were excised from bulbs and were cut into laterally. Each explant was cultured in a test tube.

Sterilization: *Lilium* bulb scales were washed in running tap water and then they were surface sterilized with 96% ethanol for 2 min, then in 2.25% Na- hypochlorite solution, with one drop of 0.1 % Tween 80 for 20 min and rinsed 4 times with sterile distilled water. In order to prevent bacterial contaminations which may occur in culture after surface sterilization streptomycin and penicillin antibiotics (Application I-A, I-B) were used. Also in order to prevent fungicide contaminations which may occur surface sterilization Benomyl and Nystatin fungicides (Application III-A, III-B) were used. In addition 300 mg/L Streptomycin + 75 mg/L Benomyl and 600 mg/L Streptomycin + 150 mg/L Benomyl with fungicide and antibiotic mixture solutions were also tested.

Application I-A: 200 mg/L Streptomycin + 200 mg/L Penicillin

Application I-B: 400 mg/L Streptomycin + 400 mg/L Penicillin

Application II-A: 300 mg/L Streptomycin + 75 mg/L Benomyl

Application II-B: 600 mg/L Streptomycin + 150 mg/L Benomyl

Appliation III-A: 50 mg/L Benomyl + 50 mg/L Nystatin

Application III-B: 100 mg/L Benomyl + 100 mg/L Nystatin

Bulb scales after surface sterilization dipped 30 min solution which contained antibiotic and fungicide combinations mentioned above.

Culture: The sterilized explants were cultured on the MS medium [25] supplemented with 8 g/L agar, 30 g/L sucrose and 0.1 mg/L NAA + 0.01 mg/L BA. The pH of the medium was adjusted to 5.7 before autoclaving at 121 °C the pressure of 1.5 kg/cm pressure for 15 min. Experiments which were experienced 16 hours light - 8 hours dark, lighting was supplied from white fluorescent lights approximately as 1600 lux, and also was experienced continuously darkness. The temperature of the culture room was changed between 19 °C and 22 °C.

Statistical Analysis: The analysis of the data was performed by Statistical Packages for Social Sciences (SPSS) 10.00 version [26].

3 Results and Discusion

Bulb formation

The best results on the root and leaf growing beside being created bulblets from explants were observed Benomyl + Nystatin applications. In explants which were cultured in photoperiodic condition and dark condition beside formation bulblet, root leaf, callus and direct shoot proliferations were seen and while in photoperiodic condition the first bulblet formation was observed 17 days later, in dark condition it was observed 19 days later. When the first bulblet formation was observed after 16 days of culture in application III- B and II-B, the last bulblet formation were seen 21 days later in all the applications.

In this study, we have developed efficient protocol for micropropagation to get a large number of bulblets from bulb scales. Cultures containing each applications were both searched in photoperiodic condition and in whole dark conditions. Bulblet formation (%) in photoperiodic condition with average 97.4 % was supplied the highest in 100 mg/L Benomyl +100 mg/L Nystatin applications (Application III-B), (Figure 1). It was 75.7 % in control group. In bulb scale explants which were cultured in whole dark conditions the highest bulb formation (%) being 83.3 in the same applications was observed (100 mg/L Benomyl + 100 mg/L Nystatin). This rate was 80 % in control (Table 1). In table 1 the bulblet formation was given for all the combinations bulblet formation (%) in photoperiodic condition out of control in all the applications was found higher than according to the dark condition. By keeping fixed applications when the importance between the applications in photoperiodic condition and dark condition 200 mg/L Streptomycin + 200 mg/L Penicillin in photoperiodic condition while bulblet formation was 88.6 % in dark it was seen as 64.9 %. This difference was found significant as statistically ($p< 0.05$). In the same way in 400 mg/L Streptomycin + 400 mg/L Penicillin application while bulblet formation rate was 88.2 % in photoperiodic condition in dark condition it was seen as 66.7 % and found significant as statistically ($p<0.05$). In other applications the difference between the bulblet formation in photoperiodic condition and dark condition wasn't significant as statistically ($p>0.05$), (Table 1). In control between photoperiodic condition and dark condition statistically there wasn't a significant difference, it was seen that bulblet formation % was better in dark condition. Although a significant difference wasn't seen in the combination of between Benomyl and Nystatin and Streptomycin in these applications in photoperiodic condition it was observed that bulblet formation % was higher.

According to the applications significant test of the bulblet weight (g/explant) of the bulb scale explants of micropropagation *Lilium candidum* was performed by variances analyze. In variances analyze to square root transformation was performed to the samples of the bulblet weight (g/explant). When all the combinations were examined significant differences were found and they divided into 4 groups. The difference wasn't seen in the group. It was entirely by change but among the groups a significant difference was seen. At the best applications from the point of view of bulblet weight

(g/explant) was observed at the 100 mg/L Benomyl + 100 mg/L Nystatin solution of photoperiodic condition. As a result of variance analyze on the weight of the bulblets from the point of applications a significant difference was noticed. It was seen that the first group was performed from the antibiotic and fungicide applications (Benomyl Nystatin and Streptomycin). Control groups were existed in the lest 2 groups among these 4 groups and it was seen that dark condition was more suitable and in addition from the point of view of bulblet weight the control group gives better results (Table 2).

Figure 1. Micropropagation of *Lilium candidium* L. (a-d). Effect of Benomyl (100 mg /L) + Nystatin (100 mg/L) solution *Lilium* root and bulb formation (a-c). Plantlets were transplanted into individiual pots (d).

Table 1. The effect of photoperiodic-dark conditions on bulblet formation (%) at different applications (*p<0.05).

Applications	Bulblet formation (%)		X^2
	Photoperiodic condition	Dark condition	
Control	75.7	80.0	0.209
Streptomycin+ Penicillin (App I-A)	88.6	64.9	5.604*
Streptomycin+ Penicillin (App I-B)	88.2	66.7	4.338*
Streptomycin+ Benomyl (App II-A)	84.8	82.1	0.081
Streptomycin+ Benomyl (App II-B)	81.1	81.3	0.000
Benomyl+ Nystatin (App III-A)	85.7	79.4	0.48
Benomyl+ Nystatin (App III-B)	97.4	83.3	Fisher

App I-A: Streptomycin (200 mg/L) + Penicillin (200 mg/L), **App I-B:**Streptomycin (400 mg/L) + Penicillin (400 mg/L) **App II-A:** Streptomycin (300 mg/L + Benomyl (75 mg/L), **App II-B:** Streptomycin (600 mg/L) + Benomyl (150 mg/L), App **III-A:** Benomyl (50 mg/L) + Nystatin (50 mg/L), **App III-B:** Benomyl (100 mg/L)+ Nystatin (100 mg/L), (*p<0.05)

Table 2. In applications bulblet weights (g/explant) of square root transformation (Duncan multiple' test)

Photoperiodic-dark condition	Applications				
Photoperiodic condition	Application III-B	.8357 a			
Photoperiodic condition	Application II-B	.8341 a			
Dark condition	Application III-A	.8072 a			
Dark condition	Application III-B	.8041 a			
Photoperiodic condition	Application II-A	.7476	.7476 ab		
Dark condition	Application I-A	.7243	.7243 ab		
Dark condition	Application III-A	.7022	.7022 ab		
Photoperiodic condition	Application I-A	.6852	.6852	.6852 abc	
Dark condition	Application I-B	.6839	.6839	.6839 abc	
Photoperiodic condition	Application I-B	.6834	.6834	.6834 abc	
Dark condition	CONTROL	.6753	.6753	.6753 abc	
Dark condition	Application II-B	.6529	.6529	.6529	.6529 abcd
Photoperiodic condition	CONTROL		.5910	.5910	.5910 bcd
Dark condition	Application II-A		.5709	.5709	.5709 bcd

Different letters in same column represent significant differences by the Duncan's multiple range test, (p<0.05).

Table 3. According to the applications without contamination rate (%)

Photoperiodic-dark condition	Applications	Non contamination (%)	Adjusted Residual (-1.96, 1.96)
Photoperiodic condition	Control	92.5	1.8
	Application I-A	87.5	1.0
	Application I--B	87.2	0.9
	Application II-A	84.6	0.5
	Application II-B	92.5	1.8
	Application III-A	87.5	1.0
	Application III-B	95.0	2.2
Dark condition	Control	100	3.1
	Application I-A	92.5	1.8
	Application I-B	76.9	-0.8
	Application II-A	70.0	-2.0
	Application II-B	80.0	-0.3
	Application III-A	89.5	1.3
	Application III-B	75.0	-1.1

Contaminations

Contaminations were seen one week later after putting the explants in culture. According to the applications contamination aspect statistically was appreciated with X^2 test. In Table 3, according to the applications without contamination explant rates were given. Among the applications, the result of the X^2 test being performed, from the point of view of contamination a significant difference was found (X^2= 107,908, $p<0.05$). The applications which create this significance, were photoperiodic condition 100 mg/L Benomyl + 100 mg/L Nystatin (95.0 %), dark condition control (100 %) and dark condition 300 mg/L Streptomycin + 75 mg/L Benomyl 80 % (Table 3).

Choosing explant in micropropagation is very important for *Lilium* cultivars. Bulb scales and leaves are being used successfully as explant [2]. In this study, bulb growing was observed in all the explants being divided lateral of bulb scales (basal, middle and terminal point) and as explant 5- 10 mm bulb scales sizes were used. Jeong (1996) mentioned that the best result was observed from basal part from bulb scales in *Lilium concoler* var parthenion [7]. *In vitro* studying the importance of the growing regulators which are supplemented with the medium is very high. Bürün et al (2002) indicated that for micropropagation of *Lilium candidium* was used different plant growing regulators which were different doses [27]. They mentioned that the most suitable plant regulator was 0.1 mg/L NAA and 0.01 mg/L BA supplemented with MS medium. So in this study, the best dose which was investigated by Bürün et al. (2002) was used. In this study showed that the highest number of bulb formation (97.4%) was obtained in the this MS medium and after surface sterilization the best application was Benomyl + Nystatin solution (Table 1). Benomyl as a systemic fungicide has the same effect as cytokinin [28]. The author reported that when different doses (10, 25, 50, 100 and 250 mg/L) of benomyl were added in culture medium, although at low doses (10 and 50 µg/L) benomyl addition were observed effective development, at high levels (100 and 250 µg/L) benomyl addition affected both root and shoot formation unfavourably and it also caused an abnormally short and thick shoot development [28]. A convenient protocol for *in vitro* propagation of *Lilium caratum* from bulb scale has been reported on MS medium supplemented with 0.1 mg/L NAA and 10 mg/L kinetin under continuously light [29](Takayama and Misawa, 1983). Maesota et al., 1994 showed that *in vitro* on bulb regeneration from bulb scale explants of *Lilium japonicum* thumb. was effect of plant growing regulators and culture conditions[30]. Dapkuniene et al., 2001 reported that the best result bulb formation was 0.1 mg/L NAA + 1 mg/L 2- IP supplemented with MS medium from some *Lilium* bulb scales to propagation scales, and also indicated that they used 5 mg/L BAP and NAA supplemented with MS medium by micropropagation in this way plantlets was enabled material from virus-free [31]. Although the addition of 0.1 mg/L BA + 0.1 mg/L NAA had no significant effect on the number of bulblets from bulb scale culture in *L. rubellum* [18], the addition of the hormones (more than 0.1 mg/L NAA with or without BA) decreased the number of bulblets in Korean lilies bulb scale culture [7]. When MS supplemented with 0.1 mg/L NAA+ 0.1 mg/L BA and high concentration of sucrose (9%) increased the number of bulblets [11], the *in vitro* bulb diameter was the largest on MS supplemented with 0.2 mg/L BA and the number of roots was the highest on 1⁄2 MS medium supplemented with 0. 2 mg/L NAA [32]. Another study which was reported have used the same plant growth regulators with our study indicated that the highest percentage of bulblet formation was observed on the medium containing 0.5 mg/L BA+ 0.5 mg/L NAA [33]. In our study, we searched environment condition 16 hours daylight- 8 hours dark to photoperiodic condition and wholly dark condition. The best result % bulb formation was observed from photoperiodic condition (Table 1). There were also other studies which indicated that the better results photoperiodic condition than dark condition in bulb growing [7,30].

In our study, the best applications from the point of view of bulblet weight (g/ explant) photoperiodic condition, 100 mg/L Benomyl + 100 mg/L Nystatin application was occupied. As a result of variance analyze on the weight of the bulblets from the point of applications and the scale of the explant a significant difference was noticed. The other study using in the same plant growth regulators with our study, addition of NAA (0.1 or 1 mg/L) and BA (0.1 mg/L) significantly increased the fresh weight of bulblets in *L. rubellum* [18].

In this study, searched as environment condition 16 hours daylight–8 hours dark to photoperiodic condition and wholly dark condition from the point of bulb weight (%) it was observed that photoperiodic condition gives better result (Table 3). There were also other studies which indicated that in bulb growing photoperiodic condition gives better results than dark condition [7,30]. *In vitro* studies beside medium and growing regulators, the temperature of the culture room effects the success also. In this study the temperature of the

culture room changed between 19- 22 °C and in this temperature successful results were got from the culture. There were some studies which show that the most suitable temperature was 20- 25°C [34] and even in 25°C temperature becoming regenare and growing bulbs adapted at greenhouse was better [35] . In our study 16 days later the first bulb appearing was seen and following this beside bulb formation, root and direct shoots were observed. Bulb existing from bulb scales in MS supplemented with 1 mg/L BA and 0.5 mg/L BA was observed 10 days later [36]. In the plant tissue culture studies under culture conditions pH is important. In the bulb scale cultures *Lilium concolor* for bulb formation, was indicated that the best result was at the 5.5 from ph levels between 3.5 and 7.5 they tried [37]. In our study also pH of the basic MS medium was adjusted to 5.7 and observed successful results.

For micropropagation among the applications searched in this study, after surface sterilization, the best application was to wait in Benomyl (100 mg/L) + Nystatin (100 mg/L) solution, and according to of bulb weight growing was better from small explants (Application III-B). In this study, in whole applications each regenerated plantlets which had green leaves and, bulbs and roots was transferred to pot nearly two mounts later (Figure 1). Saifullah et al. (2010) reported that plantlets grown for 3-4 weeks *in vitro* transferred to pots and indicated that sterile healty roots showed 100 % survival in greenhouse [4].

4 Conclusion

In this study, as alternative to vegetative reproduction a suitable method was performed to give opportunity to reproduce L. candidum with micropropagation and at the end of the study the bulb growing % was observed higher from bulb scale explants which in the photoperiodic condition than in the dark condition (Table 1). After surface sterilization the best application to be performed was by the help of the statistically analyzer both from the point of bulb growing and the weight of the bulb (bulb weight) determined that it was Benomyl (100 mg/L) + Nystatin (100 mg/L) and also the point of view the number of the bulblet the best application was obtained Benomyl + Nystatin solution. As a result it was found that all the applications for micropropagation of *Lilium candidum* 16 hours day light - 8 hours dark photoperiodic condition, the sterilization of explant and bulb scales before culturing must be immersed in Benomyl + Nystatin solution (Table 1), (Figure 1).

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