

## Influence of different sterilization methods on callus initiation and production of pigmented callus in *Arnebia densiflora* Ledeb.

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**Abstract:** We analyzed the effects of sodium hypochlorite and Plant Preservative Mixture (PPM) on surface sterilization. We also examined the effects that the addition of an antibiotic-antimycotic solution to the culture medium had on callus induction. Explants were initially sterilized with different concentrations of sodium hypochlorite and cultured on MS media containing kinetin (0.29  $\mu$ M) and naphthalene acetic acid (NAA, 10  $\mu$ M). No calluses were produced, either because of contamination of the explants, or loss of explants as a result of the high levels of sodium hypochlorite. The application of PPM and antibiotics at different concentrations reduced contamination and led to callus induction from shoot apices and young root explants. The best callus responses were obtained using PPM at 1%-2%, whereas callus induction on shoot apices diminished at higher concentrations (4% PPM). This is the first report of successful sterilization and reduced contamination of explants from naturally field grown *A. densiflora* by PPM. Moreover, established callus cultures produced pigmented calluses, which were analyzed spectrophotometrically.

**Key words:** *Arnebia densiflora*, sterilization, PPM, sodium hypochlorite, antibiotic-antimycotic solution

### *Arnebia densiflora* Ledeb.'de kallus oluşumunda farklı sterilizasyon metodlarının etkisi ve renkli kallus üretimi

**Özet:** Bu çalışmada sodyum hipoklorit ve Bitki Koruyucu Karışımın yüzey sterilizasyonundaki etkileri araştırılmıştır. Antibiyotik-antimikotik çözeltisinin kültür ortamına eklenerek kallus indüksiyonundaki etkilerini inceledik. Eksplantlar başlangıç olarak farklı sodyum hipoklorit konsantrasyonlarıyla steril edilerek kinetin (0,29  $\mu$ M) ve naftalen asetik asit (NAA, 10  $\mu$ M) içeren MS ortamında kültüre alınmıştır. Eksplantlardaki kontaminasyonlar veya yüksek sodyum hipoklorit konsantrasyonundan dolayı kallus oluşumu gözlenmemiştir. Farklı konsantrasyonlardaki Bitki Koruyucu Karışımı ve antibiyotikler kontaminasyonu azaltmış ve sürgün ucu ve genç kök eksplantlarında kallus indüksiyonu gerçekleşmiştir. En iyi kallus oluşumu % 1-2 Bitki Koruyucu Karışım kullanımında bulunurken, sürgün uçlarındaki kallus indüksiyonu yüksek konsantrasyonda (% 4) azalmıştır. Bu çalışma, doğal ortamında arazide yetişen *A. densiflora*'nın eksplant kontaminasyonlarının azaltılması ve başarılı sterilizasyon ile ilgili ilk çalışmadır. Oluşturulan kallus kültürleri renkli kalluslar üretti bunlar da spektrofotometrik yöntemle analiz edildi.

**Anahtar sözcükler:** *Arnebia densiflora*, sterilizasyon, bitki koruyucu karışım, sodyum hipoklorit, antibiyotik-antimikotik solusyon

## Introduction

*Arnebia densiflora* Ledeb. is a perennial species that is endemic to Turkey. The roots, and specifically the root cortex, of this species contain more alkannin/shikonin derivatives than those of other members of the family Boraginaceae. The pink-red colored roots of the plant contain naphthoquinones, and have been used in skin care preparations, as a colorant in cosmetics, and in the food and textile industries. The production of these naphthoquinone pigments has been hindered by contamination of plant tissue cultures derived from roots and velutinous plant organs. Contamination of plant tissue cultures by different sources, such as bacteria and fungi, reduces their productivity and can completely prevent their successful culture. Several different methods are used to eliminate fungal and bacterial contamination, including the use of antibiotics, fungicides, and inactivation by heat and light (1-6). Sodium hypochlorite (NaOCl) and calcium hypochlorite are the main surface sterilizing agents used in plant cell and tissue culture experiments. Following surface sterilization, explants are either submerged in an antibiotic-antimycotic solution, or these agents are added to the culture medium to prevent the growth of microorganisms on the explants (2,3).

Plant Preservative Mixture (PPM) is a biocide containing methylchloroisothiazolinone, methylisothiazolinone, magnesium chloride, magnesium nitrate, potassium sorbate, and sodium benzoate. It has a broad spectrum and is able to prevent the growth of microorganisms when used for surface sterilization in concentrations of up to 4 mL/L, with minimal effects on germination and tissue culture studies (7). PPM eradicates microorganisms by targeting the enzymes in the Krebs cycle and the electron transport chain (7). In order to sterilize heavily contaminated explants, PPM can either be used to immerse explants, or be added to the culture medium at different concentrations. In citrus species, 0-2 mL/L of PPM were added to the medium with no negative effect on callus induction or shoot organogenesis, whereas the same concentrations were recorded as toxic in protoplast cultures (8). In another study, the addition of 5 mL/L of PPM to the medium was effective in preventing the contamination of explants obtained from greenhouse and field grown

citrus trees, previously sterilized by the usual surface sterilization methods (9). Further studies have shown that, depending on the plant species, PPM can be used at different concentrations with no negative effects on plant growth. For example, up to 10 mL/L of PPM was applied with no adverse effects on tobacco androgenesis, whereas concentrations above 5 mL/L and 2 mL/L inhibited melon somatic embryogenesis and petunia adventitious shoot organogenesis, respectively (10). In addition to its use in plant tissue culture media, surface sterilization of seeds has been achieved with 4% PPM (11). The results of some studies suggested that seed could be sterilized at various concentrations of PPM, depending on the plant species (12-14), while other studies (in transgenic arabidopsis plants for a Space Shuttle flight experiment and bryophytes) found PPM to be ineffective (15,16).

A variety of active drug constituents can be produced by utilizing plants and plant tissue culture techniques in vitro. The induction of calluses and the establishment of suspension cultures are the basic in vitro methods used to produce active phytochemicals. *A. densiflora* Ledeb. is a medicinal plant used in folk medicine for wound healing purposes, which is also proved scientifically (17). The plant, specifically the root and root cortex, produces dark pink-red colored shikonin and its derivatives, which are naphthoquinones. In the Far East, particularly in Japan, *Lythospermum erythrorhizon* has been used to produce shikonin and its derivatives via tissue culture methods since the 1980s. Shikonin and its derivatives have been shown to possess a variety of pharmacological and biological activities including antitumor, antiinflammatory, antimicrobial, antioxidant, and antiHIV-1 activities (18-21). The *Arnebia* species, especially *A. densiflora*, has the highest shikonin level among the other shikonin-producing species (22).

This perennial plant has green-yellow leaves and stems covered with hairs, which are the main problem when conducting aseptic studies. Moreover, the plant naturally grows in Turkey on selected hills in very limited regions where the soil is rich in minerals, making the plant very difficult to grow in greenhouse conditions (23,24). In addition, the roots, which are the main source of explants and the primary shikonin

derivative producing organs, are the most difficult part to sterilize. Serious contamination problems are also associated with explants derived from the hairy leaves and stems. Conventional sterilization methods, such as rinsing the explants with 70% ethanol followed by the application of NaOCl at different concentrations, have been used in attempts to reduce contamination. However, these methods were ineffective, with a contamination recurrence rate of 98%. In this study, we compared the effects of using different sterilization methods and materials, such as NaOCl, antibiotic-antimycotic solution, and PPM on *A. densiflora* explants, and also compared the effects of these compounds on callus induction.

## Materials and methods

**Plant material.** Flowering *A. densiflora* Ledeb. plants were collected from Sivrihisar Aşağı Kepen town, Eskişehir, Turkey, in May-August. Shoot apex (0.1-0.5 cm) and root (0.5-1 cm) fragments were used as explants.

**Sterilization.** All explants were incubated in 70% ethanol for 1 min and then transferred to the sterilization solutions. NaOCl and PPM were used for surface sterilization. A group of control explants were rinsed with 70% ethanol and incubated in sterilized, autoclaved water.

For surface sterilization, 1.25%, 1.5%, 1.75%, and 2% solutions of NaOCl (ACE, Turkey commercial NaOCl was used as a stock in the sterilization process, 5% NaOCl) were prepared with the addition of 1 drop of Tween 20. Aqueous solutions of 1%, 2%, 3%, and 4% PPM were prepared. Explants were incubated for 20 min in each concentration of both sterilizing agents. All explants were then washed in autoclaved distilled water.

The antibiotic-antimycotic solution consisted of the antifungal agent, amphotericin B (Fungizone, 50 mg, Bristol-Myers Squibb, Princeton, NJ, USA), penicillin G (sodium salt, Sigma, St. Louis, MO, USA (69-57-8) P-3032, 100 MIU), and streptomycin sulfate (BiochromAG A-331-27752 mg Berlin, Germany). The experimental solutions with final concentrations of 250, 500, and 1000 mL/L were prepared from these stock solutions and added to the media after filter sterilization using a 0.25 µm filter. The explants were surface sterilized with 25% NaOCl

for 20 min, rinsed with distilled, autoclaved water, dried, and then placed on media containing added antibiotic-antimycotic solution. Contamination was evaluated 15 days after the first incubation. Numbers of contaminated explants were counted.

**Callus induction.** Shoot apex and root fragments were initially sterilized as described above and then transferred to MS (25) media containing 0.29 mM kinetin and 10 mM NAA (26,27), at a pH of 5.7. All explants were inoculated in the dark at 22-24 °C and were transferred to fresh media every 30-35 days.

**Statistical analysis.** Five explants were placed into each petri dish. Thirty explants were established in each treatment. Each treatment was replicated 3 times. After 4 weeks, the calluses formed on explants were counted. The percentage of callus formation was determined. Data were subjected to one-way analysis of variance (ANOVA) and the differences among means were compared using Duncan's multiple-range test (28). The treatments were arranged in a completely randomized design. Data given as percentages were subjected to arcsine transformation (29) before statistical analysis.

**Spectrophotometric analysis:** For this, 0.4 g of dried root cortex and callus cultures were extracted with chloroform. Extracts were evaporated and added to 10 mL of chloroform. Then 1 mL of extract was scanned at 400-700 nm in a Beckman Du-600 spectrophotometer. Shikonin and its derivatives gave peaks at 580 and 620 nm (30). The shikonin derivatives' content was calculated by using the calibration curve that was previously calculated by using shikonin standard.

## Results and discussion

All explants in the control group were contaminated after a few days, giving a contamination rate for the control group of 100% by day 15 (Figure 1). After 15 days, almost all the explants in low concentrations of NaOCl were contaminated (10%-15%), whereas in high NaOCl concentrations (35%-40%), nearly all the explants lost their viability and very few calluses were formed (1%-2%). However, the application of PPM and antibiotics was effective and callus initiation was observed on explants originating from shoot apices and roots (Figure 2).



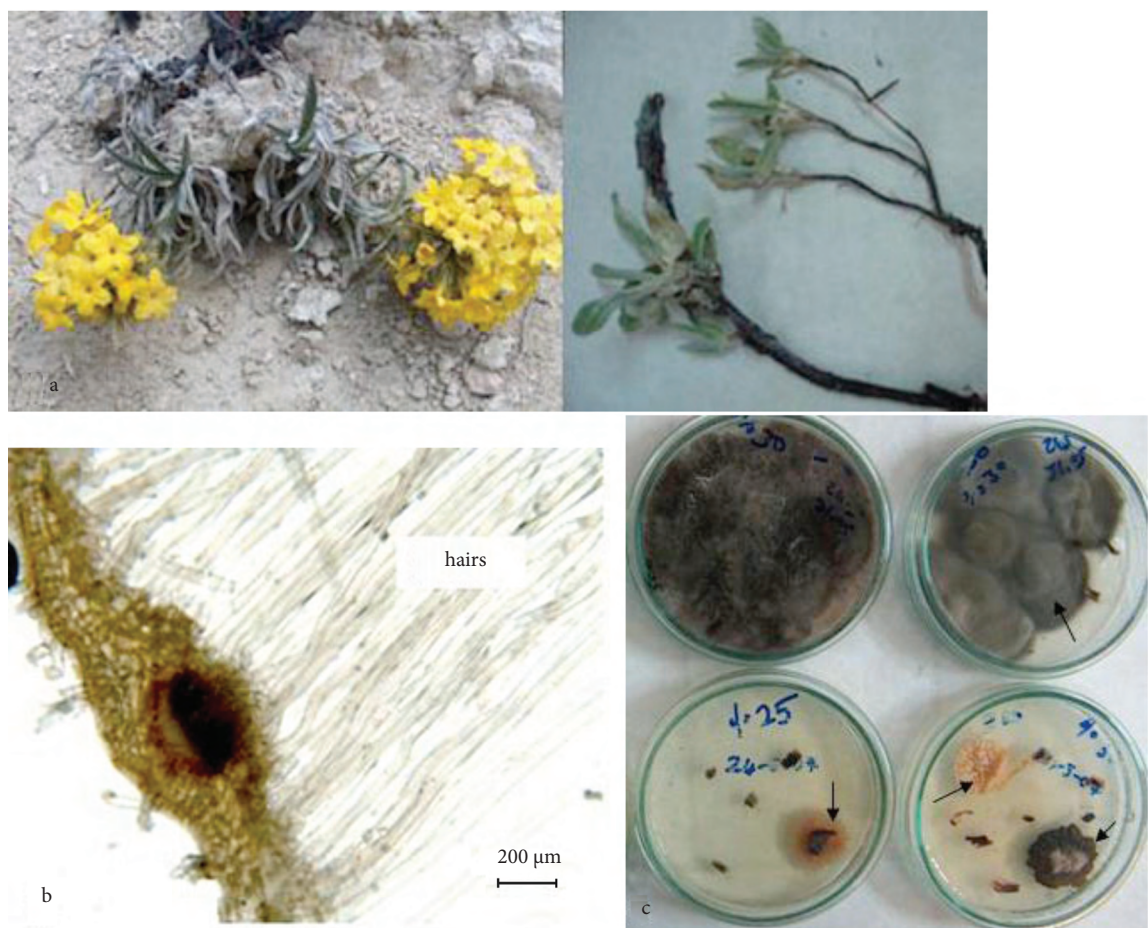


Figure 1. a) *A. densiflora* in habitat (left), and shoots, roots (right) b) Velutinous leaf of *A. densiflora*, c) Contamination originating from explants.

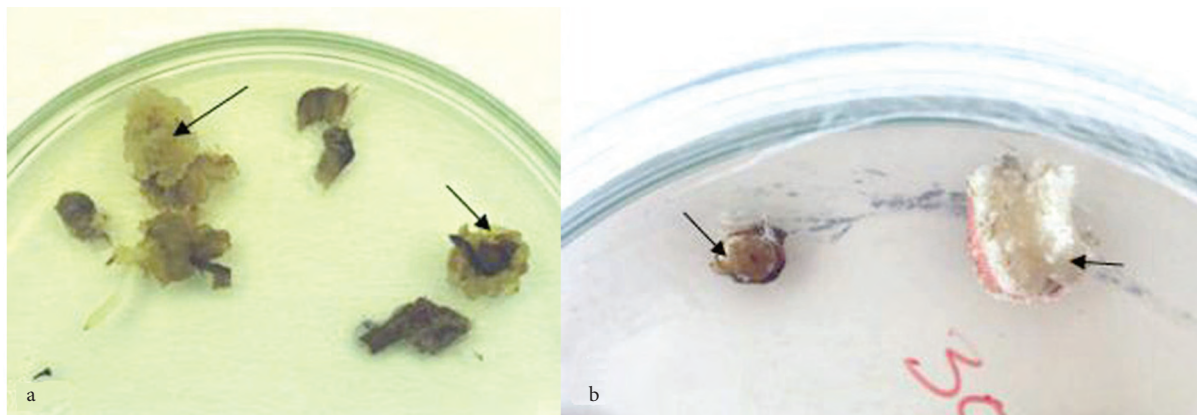


Figure 2. Callus formation on shoot apex of *A. densiflora* (a), emerging callus on root explants of *A. densiflora* (b).

The best callus response from the shoot apex explants was obtained using PPM at concentrations of 2%-3%, whereas the best callus response from root explants was obtained with 2% PPM (Figure 2). Contamination decreased with an increase in PPM concentration, but callus initiation also decreased with the elevation of the PPM concentration. For example, successful callus development occurred at up to 3% PPM, but the callus initiation rate decreased at 4% PPM (Table).

Application of the antibiotic combination resulted in successful callus induction on young shoot explants, but not on root explants. An antibiotic concentration of 250 mL/L had no effect on the contamination rate, but contamination decreased gradually at concentrations of 500 and 1000 mL/L. Although the highest rate of callus development occurred on media with the 500 mL/L antibiotic combination, there was no significant difference in callus formation rates at the different

concentrations. Antibiotics had no significant effect on contamination of root explants, which was more effectively treated with PPM.

Besides sterilization studies, different media compositions such as LS, SH, and MS were tested to produce the best callus formation (25). The best friable grayish-white callus was obtained with MS media. Since the roots of the plant produced the naphthoquinone pigments, calli cultures were incubated in the dark. Twenty days after the transfer of the calli cultures to the fresh media, red dots were observed on the calluses of only a few plates. Later on, different shades of red and pink-burgundy calluses were observed (Figure 3). These pigmented calli cultures were collected, freeze dried, and analyzed by spectrophotometer to confirm if these pigmented callus were producing naphthoquinone derivatives. Shikonin derivatives (naphthoquinones) were detected spectrophotometrically in pigmented callus cultures quantitatively as 1061 µg/g.

Table. Contamination and callus initiation rates on shoot and root explants after different sterilization methods.

Sterilization agents	Shoot explants		Root explants	
	Contamination (%)	Callus (%)	Contamination (%)	Callus (%)
PPM (%)				
1	23.33 ± 8.8a	23.17 ± 5.2a	28.33 ± 4.4a	5.53 ± 2.2a
2	15.61 ± 4.4ab	25.00 ± 8.7a	24.43 ± 2.9a	13.86 ± 5.6a
3	8.33 ± 1.7bc	25.00 ± 2.9a	21.27 ± 0.7a	8.50 ± 1.5a
4	0.00 ± 0c	13.33 ± 8.8b	4.17 ± 3.0b	5.00 ± 2.5a
Antibiotic-antimycotic solution (mL)				
250	69.39 ± 0.9a	11.43 ± 7.7a	97.73 ± 1.5a	0.10 ± 0.1a
500	29.82 ± 10.6b	20.31 ± 7.5a	90.91 ± 7.2a	3.64 ± 2.0a
1000	27.05 ± 8.7b	15.71 ± 7.8a	79.11 ± 11.5a	2.38 ± 2.3a

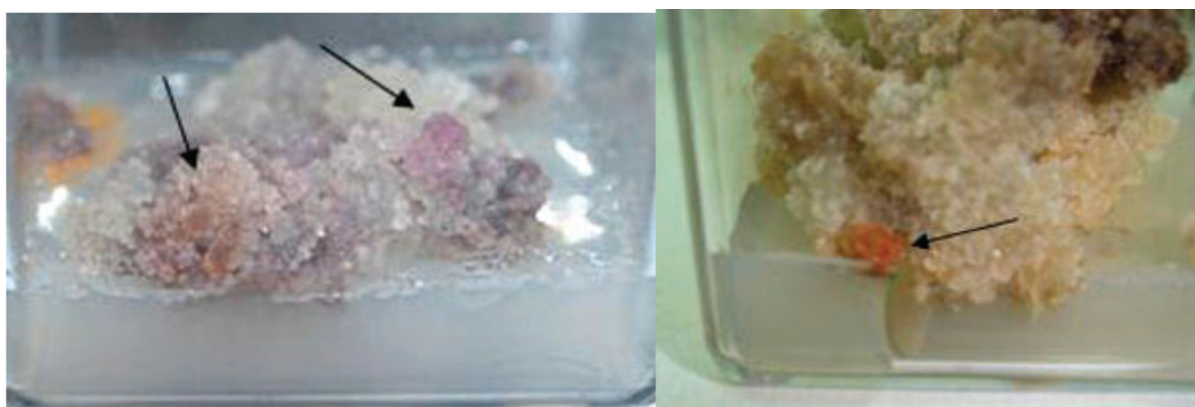


Figure 3. Pigmented callus formation on root callus of *A. densiflora*.

In order to eliminate contamination, *Arnebia* explants were sterilized with PPM and antibiotic-antimycotic solutions. PPM was more effective than the antibiotic-antimycotic solution in both the shoot apex and root explants. On the other hand, PPM inhibited callus induction at high concentrations in all explants. In previous studies, PPM has been used for the surface sterilization of seeds, while other researchers have added it to the culture medium to prevent contamination originating from the explants. Because *A. densiflora* has stubborn hairs covering its aerial parts, PPM was used for surface sterilization in this study, rather than as an addition to the medium.

In order to sterilize *Arabidopsis thaliana* seeds, Solfanelli et al. used an overnight incubation with 4% PPM, and the addition of 0.05% PPM to the medium did not produce any negative effects (11). Most studies have added PPM to the culture medium at lower concentrations. Guri et al. added 0.4% PPM to the medium in order to prevent the contamination of cucumber seeds. Concentrations of >0.4% reduced the germination rate (7). Another study examined the effects of PPM on callus formation on melon embryos and embryo production. No side effects were reported when 0%-0.5% PPM was used, whereas callus formation and embryo production were suspended at concentrations >0.5%. Tobacco androgenesis, however, was unaffected by PPM concentrations up to 1%, but was inhibited at higher concentrations (10).

In this study, the best results were obtained with the application of 2%-3% PPM to the shoot apex

and 1%-3% PPM to the root explants, resulting in the development of yellowish-white calluses. Applications of PPM at concentrations >3% to the shoot apex and >2% to the root explants reduced callus formation. The production of calluses from the roots has previously been difficult due to heavy contamination problems, but the use of PPM in this study led to successful root callus formation.

In addition to PPM, we also investigated the effects of an antibiotic-antimycotic mixture on the sterilization of the explants. The application of antibiotics and antimycotics has produced successful results in previous studies. A mixture of Benlate, cefotaxime (5 mg/mL), and vancomycin (0.25 mg/mL) reduced contamination by an average of 30%-35% (31). In a different study, penicillin G and streptomycin, both at 1000 mg/mL, were effective in eliminating bacterial contamination on ginseng, but caused abnormal and depressed callus formation and inhibition of somatic embryogenesis (32).

In the present study, streptomycin, penicillin G, and amphotericin were mixed and used at concentrations of 250, 500, and 1000 mL/L. In a study with chrysanthemum and tobacco plants, increasing concentrations of antimycotic had negative effects on shoot formation (33), whereas in the current study no such inhibition was observed, and indeed the mixture slightly increased callus induction from the shoot apex and roots between 250 and 500 mL/L. Although the antibiotic-antimycotic mixture reduced the level of contamination, it did not eliminate it completely. The application of 25%



NaOCl for surface sterilization, prior to the use of the antibiotic-antimycotic mixture severely reduced callus formation.

According to the results of this study, PPM at 2%-3% was more effective at reducing contamination of *A. densiflora* explants, specifically on shoot explants, than the tested antibiotic-antimycotic mixture. The application of different concentrations of PPM can be successfully used for surface sterilization of problematic explants, as well as for the sterilization of seeds. PPM could be a useful sterilizing agent for hairy and heavily contaminated explants collected from nature, which need to be maintained in aseptic conditions.

Established callus cultures also produced pigmented compounds in this study. The data showed that the pigmented calli were producing naphthoquinone derivatives, mostly alkannin/shikonin. Only these reddish-pink calli cultures were collected and were transferred to obtain a naphthoquinone producing cell line, but the callus did not stay colored, after each transfer, the color slightly changed to grayish-white. Although pigmented compounds were obtained in the cultures, what the influences were that cause the callus to produce color compounds is still not completely solved. Analysis

of the phytochemical content of callus cultures has been under investigation. Studies are also ongoing to produce stable reddish pink callus or suspension cultures by applying different media or elicitors.

To the best of our knowledge, this is the first manuscript about the usage of PPM on the sterilization of *A. densiflora* explants, as well as on the production of naphthoquinone pigmented callus culture.

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