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# Optimization of in vitro sterilization for pistachio (*Pistacia vera* L.) rootstocks

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# Antep fıstığı (*Pistacia vera* L.) anaçlarında in vitro sterilizasyon optimizasyonu

**Abstract:** Pistachio (*Pistacia vera* L.) is one of the leading edible nut consumed all over the World due to its nutritional values. The plant is cultivated in most of the countries along with Türkiye which is one of the leading grower of pistachio. In Türkiye, the rootstock material is currently propagated through traditional methods and there is a need of propagating plant material using modern biotechnological techniques like plant tissue culture. The provision of contaminated free explants with minimum or no phenolic compounds in the culture medium is the prerequisite of *in vitro* regeneration protocol. The plant material used in this study was collected at different physiological stages during different months like April- June and Sep-October. The plant material was cut into 2-3 cm long nodal segments followed by cleaning with different agents like water, soap and fungicide prior to subjected to sterilizing agents. Different sterilizing agents used in this study were $HgCl_2$, Huwa-san ($H_2O_2$) and commercial bleach (NaOCl) for both rootstocks (UCB-1 and Buttum) with different exposure time. Sterilized explants were cultured on MS basal medium containing plant growth regulators and sub-cultured once a week for three weeks. Results revealed that $HgCl_2$ as sterilizing agent was more superior than other sterilizing agents for both rootstocks. Among rootstocks, UCB-1 was more responsive than Buttum and relatively more sterilized plants were attained. On the other hand, plant material collected during June responded better and 90.0% and 50.0% sterilized plants were attained for UCB-1 and Buttum respectively. The results revealed the significant impact of collection time, sterilizing agent type, concentration and exposure time on sterilization of *P. vera* rootstocks.

**Key words:** In vitro, pistachio, phenolics, rootstocks, sterilization

**Özet:** Antep fıstığı (*Pistachio vera* L.), besin değerleri sebebiyle dünyada en çok tüketilen kuruyemişlerden biridir. Antep fıstığı, fıstık yetiştiriciliğinde önde olan Türkiye ile birlikte birçok ülkede yetiştirilmektedir. Türkiye'de genel olarak anaç materyali geleneksel yöntemler ile çoğaltılmaktadır ve bitki materyallerinin bitki doku kültürü gibi biyoteknolojik teknikleri kullanılarak çoğaltılmasına ihtiyaç vardır. In vitro rejenerasyon protokolünün ön koşulu, kültür ortamında kontamine olmayan, minimum veya hiç fenolik bileşik içermeyen eksplantların sağlanmasıdır. Bu çalışmada kullanılan bitki materyali, Nisan-Hazıran ile Eylül-Ekim ayları arasında değişen farklı aylarda, farklı fizyolojik aşamalardayken toplanmıştır. Bitki materyali 2-3 cm uzunluğunda nod parçaları olarak kesilmiş ardından sterilizasyon ajanlarına tabi tutulmadan önce su, sabun ve mantar ilacı gibi farklı ajanlarla temizlenmiştir. Yapılan sterilizasyon çalışmada her iki anaç (UCB-1 ve Buttum) için farklı sürelerde $HgCl_2$, Huwa-san ($H_2O_2$) ve ticari çamaşır suyu (NaOCl) farklı sterilizasyon ajanları kullanılmıştır. Daha sonra eksplantlar, bitki büyüme düzenleyicileri içeren bazal ortamlarda kültürlendi ve üç hafta boyunca haftada bir kez kültür aktarıldı. Sonuç olarak, sterilizasyon ajanı olarak $HgCl_2$'nin her iki anaç için de diğer sterilizasyon ajanlarından daha üstün olduğunu ortaya koymuştur. Anaçlardan UCB-1, Buttum'a göre daha duyarlı ve nispeten daha steril bitkiler elde edildi. Sonuç olarak, *P. vera* anaçlarının sterilizasyonunda eksplant alım zamanı, sterilizasyon ajanının türü, konsantrasyonu ve maruz kalma süresinin önemini ortaya koymuştur.

**Anahtar Kelimeler:** Anaç, Antep fıstığı, fenolikler, in vitro, sterilizasyon

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## 1. Introduction

Application of biotechnological techniques like *in vitro* plant tissue culture is highly significant for producing plants at larger scale in short time especially for fruit trees and ornamental plants (Hesami and Daneshvar, 2016, 2018; Hesami et al., 2018 a,b). The other advantage of *in vitro* regeneration protocol is its application for genetic improvement of plants with elite characteristics and also for breeding purposes (Hesami et al., 2019). However, optimization of sterilization process is highly significant for the establishment of these protocols (Aasim et al., 2013). The contamination of explants is the major inhibiting factor for the development of successful regeneration protocol (Hesami et al., 2017). The occurance of contamination is due to the presence of different microorganism (endogenic and exogenic) in the explant (Arab et al., 2014; Da Silva et

al., 2016a). Therefore, sterilization process need more time, attention and techniques (Mihaljević et al., 2013; Jafari et al., 2016) in order to eliminate contamination with objective to attain explants without damage to proceed for in vitro regeneration process (Batti et al., 2020).

The optimization of whole sterilization process is dependent on variable factors ranging from plant age, size, location, physiological and enviromental factors and explant (Da Silva et al., 2016b). These factors later on regulate the in vitro regeneration process (Hesami et al., 2018c) and selection of sterilizing agent and exposure time are significant with focus on obtaining contamination free explants without substantial damage. The most commonly used sterilizing agents are NaOCl (sodium hypochlorite), $H_2O_2$ (hydrogen peroxide), $Ca(ClO)_2$ (calcium hypochlorite), $HgCl_2$ (mercury II chloride), silver based chemicals like $AgNO_3$ (silver nitrate) or NS (Nano-silver) and PPM (Mihaljević et al., 2013; Nongalleima et al., 2014). Besides that, several other commercial chemicals, antibiotics and fungicides are also employed for in vitro sterilization process (Aasim et al., 2019; Barpete et al., 2021). Another important factor in sterilization is the exposure time of explants to the specific sterilizing agents. It is already established that there is correlation between sterilizing agent, dose and exposure time on sterilization (Nongalleima et al., 2014; Hesami et al., 2018c). Therefore, it is highly important to select sterilizing agent with low phytotoxicity and high inhibition rate of contaminants (Arab et al., 2014; Jafari et al., 2016).

Türkiye is one of the leading pistachio grower (Ertürk et al., 2015) and its cultivation is mostly done in Southeastern Anatolian Region (Çağlar et al., 2017). Türkiye ranked 4$^{th}$ in the production of pistachio with annual production of 74.828 ton (FAO, 2018). In Türkiye, the production of pistachio rootstock is generally done with grafting. However, elevated demand of rootstocks in recent years forced the producers to use modern biotechnological techniques like plant tissue culture. Although, research carried out in Türkiye on pistachio revealed the successful development of in vitro regeneration protocols (Onay et al., 2004; Ozden-Tokatli et al., 2005; Kılınç et al., 2015), the commercial production of pistachio rootstocks is still neglegible due to certain issues during plant tissue culture. The major concern is the availability of contamination free explants alongwith inhibiton of phenolic compounds in the culture medium which hinders the sprouting of shoots from sterilized explants. Therfore, establishment of successful sterilization protocol is highly noteworthy for the development of in vitro regeneration protocol of pistachio. Keeping in view, the study was designed for the optimization of sterilization for two different rootstocks of pistachio commonly used in Türkiye. Plant material in this study was collected at different physiologial stages and subjected to different sterilizing agents and exposure time.

## 2. Materials and Method

The shoots with 5-10 nodes of two different pistachio rootstocks (UCB-1 and Buttum) were procured/collected from Pistachio Research Institute, Gaziantep, Türkiye. The plant material was also grafted and cultured under greenhouse conditions at Bademli Biyoteknoloji Sanayi ve Ticaret Limited Ödemiş, Izmir for the availability of continuous plant material. The plant material used in this study were collected in different months and sources (1, 2).

The leaves were removed and twigs were placed under running water to remove trashes prior to surface sterilization (Benmahioul et al., 2016). Thereafter, shoots were cut into pieces of 2-3 cm long with at least one node (nodal segment) for sterilization. Different experiments were carried out using different sterilizing agents followed by inoculation on culture mediums enriched with different plant growth regulators. In all experiments, 30 explants were used from both rootstocks for each sterilizing agent.

***Experiment 1:*** The plant material collected during April was subjected to the following procedure of washing material with 5% liquid soap (5 ml Protex+95 ml dH2O) and washed with running under tap water followed by soaking of nodal segments with 30% fungisit (MERPAN 80 WG - Captan) for 30 min and washed under running under tap water. Thereafter, material was sterilized with 0.1 % $HgCl_2$ for 55 min followed by three times washing with sterile water. After sterilization, nodes were cultured on MS medium enriched with 2 mg/L BA + 2 mg/L AgNO3. Similar sterilization procedure was also adapted for plant material collected during May with minor modificcation of using 0.1 % $HgCl_2$ for 45 min.

***Experiment 2:*** The plant material was procured during second week of September and during May. The plant material (nodal segments) were subjected to sterilization using three different sterilizing agents in this study. The overall procedure used for sterilization is given in Table 1. Three different sterilizing agents used were 0.1 % $HgCl_2$, 10.0% Huwa-San ($H_2O_2$) and 10.0% commercial bleach. After sterilization, nodal segments were cultured in glass tubes containing MS medium enriched with 2 mg/L BA + 2 mg/L $AgNO_3$. Nodal segments were subcultured once a week for 3 times and then cultured on MS medium enriched with 0.5 mg/L $GA_3$.

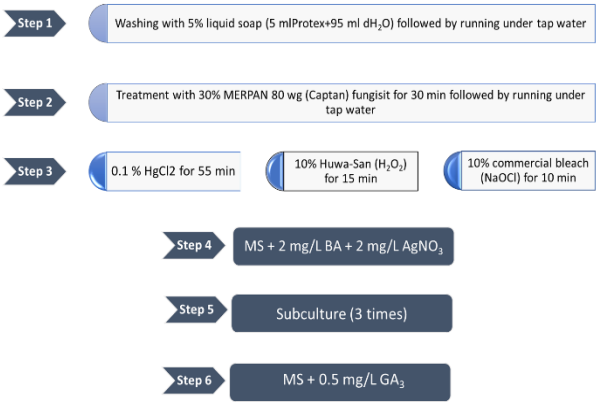


**Figure 1**: An overview of sterilization using different sterilizing agents

***Experiment 3:*** The plant material (UCB-1) collected during end of October (Pistachio Research Institute) were subjected to sterilization by cleaning the nodal segments with water and subjected to 0.1% $HgCl_2$ for 45 min and rinsed with sterile $dH_2O$. Nodal segments were transferred to MS medium enriched with different BAP (1.0, 2.0, 3.0, 4.0, 5.0 mg/L) concentration and 25 mg/L Polyvinylpyrrolidone (PVP) for 8 weeks without any subculture.

After the completion of experiment, the success of sterilization process (%) was calculated by counting and tabulated by using formula based on non-contaminated plants and results were given in percentage.

$$\text{Sterilization (\%)} = \frac{Contamination\ free\ explants}{Total\ explants}\ x\ 100$$

The basal mediums used in this study was MS (Murashige and Skoog, 1962) medium used at 4.4 g/L, 30 g/L sucrose and medium was solidified by agar used at the rate of 6.5 g/L. The pH of the medium was adjusted to 5.8 by using 1N NaOH or 1N HCl. The incorporation of $AgNO_3$ and PVP were done prior to autoclaving the culture medium. The medium was poured into glass tubes (25 mm wide) or plastic falcon tubes (15 mm wide). After placing explants into the culture medium, tubes were placed in the growthroom equipped with White cool flourescent (experiment 1 and 3) or white LEDs (experiment 2) at 16h light photoperiod. The temperature of the growthroom was adjusted at 24± 1℃.

Analysis of variance (ANOVA) of different experiments were performed by using SPSS 20.00 for Windows. Duncan's multiple range test (DMRT) was used for Post hoc tests for comparing means difference. The data was transformed into Arcsine square root transformation (Snedecor and Cochran, 1967) before statistical analysis.

**3. Results**

The results revealed the clear impact of material collection timing and sterilizing agents for sterilization process for both rootstocks. The results of experiment 1 revealed the high contamination rate (100%) irrespective of exposing explants for more time. There was no shoot induction from nodal segments explants due to high contamination and possibility of dormancy phase of axillary buds at the time of collection. Besides that extensive release of phenolic compounds also inhibited the shoot induction.

Three different sterilizing agents were employed for plant material collected at different time in experiment 2. Results clearly revealed the clear impact of collection time and sterilizing agent on sterilization process. The response of rootstock was also different with each other. The samples collected during september 2020 revealed relatively low response (Figure 2) than plant samples collected during May (Figure 3) for both rootstocks.

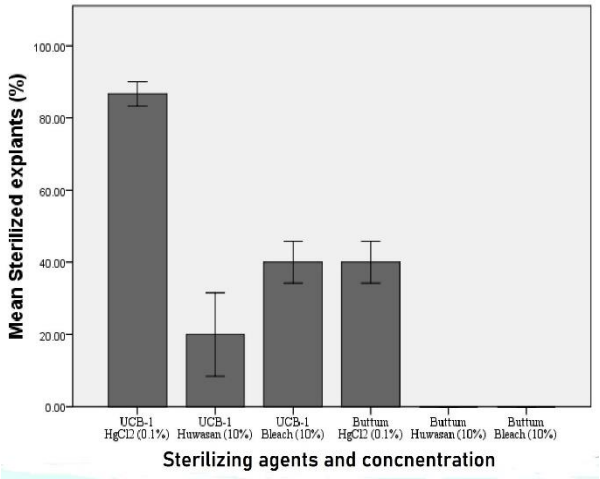


**Figure 2:** Optimization of sterilization of UCB-1 and Buttum rootstock material collected during month of September

The results given in Fig. 2 revealed that application of 0.1% $HgCl_2$ for 55 min was more effective than other treatments for both rootstocks and recorded 40.0% and 20.0% respectively for UCB-1 and Buttum rootstock. Whereas, application of 10% Huwa-San and 10% bleach resulted in 30% and 20% contamination free explants for UCB-1. Whereas, the response of Buttum was not good and 100% contamination was recorded for both 10% Huwa-San and 10% bleach. Comparison of both rootstocks revealed that UCB-1 was more responsive than Buttum. Whereas relatively more damage was associated with Huwa-san and bleach. On the other hand, plant samples collected during May were more responsive than september collected samples but follwed the same trend like samples collected during September. Application of 0.1% $HgCl_2$ resulted in relatively high non-contaminated nodal segments for both rootstocks and recorded as 87.0% (UCB-1) and 40.0% (Buttum). Results further revealed that other sterilizing agents also responded better and 20.0% (Huwa-San) and 40.0% (bleach) non-contaminated nodal segments was recorded for UCB-1. On the other hand, Buttum was less responsive to these two sterilizing agents and resulted in 100% contamination. Application of bleach resulted in damaging the explant which was more prominent in buttum compared to UCB-1. Regular subculture for three times found to be effictive to reduce the phenolic compounds. This decresae in phenolic compounds is possibly due to subculture and presence of $AgNO_3$ in the culture medium.

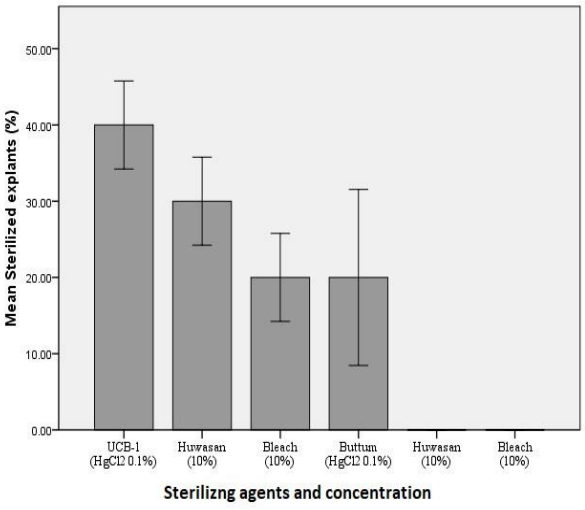


**Figure 3**. Optimization of sterilization of UCB-1 and Buttum rootstock material collected during month of May

After analysing the results of sterilizing agents and plant material collection time, another eperiment was desigend using only 0.1% $HgCl_2$ for plant material collected in the month of June. The results revelaed relatively low contamination than other experiments. The contaminated free nodal segments were recorded as 90.0% for UCB-1 and 50.0% for Buttum (Table 1). Results also revealed the relatively low phenolic compounds in the culture medium compared to other experiments. These results clearly revealed that collecting time of plant material and sterilizing agent is highly significant for obtaining contamination free explants for *in vitro* micropropagation of pistachio rootstocks.

**Table 1.** Optimization of sterilization of UCB-1 and Buttum rootstock material collected during month of June

| Rootstock | Sterilizng Agent | Mean Sterilized explants (%) |
|---|---|---|
| UCB-1 | %0.1 $HgCl_2$ | 90.00 |
| Buttum | %0.1 $HgCl_2$ | 50.00 |

Plant material collected during late October was subjected to sterilization process of using 0.1 % $HgCl_2$ for 45 min followed by culture on MS medium enriched with different BAP concentrations and 25 mg/L PVP (experiment 3). Results revealed zero contamination irrespective of culturing continuously on the same medium for 8 weeks. However, explants turned into black with the passage of time due to extensive release of phenolic compounds in the culture medium. The level of phenolic compounds in the culture medium was variable with BAP concentration and minimum phenolic compounds were recorded for MS medium enriched with 3.0 mg/L BAP+25 mg/L PVP. These results suggested that phenolic compunds can be minimized by using different culture mediums and addition of subculture could lead to minimum phenolic compounds alongwith high possibility of shoot induction. The results also suggest that contamination is dependent on collection time and plant material from where samples were taken.

## 4. Discussion

Sterilization of plant material taken directly from field or potted plants are generally difficult to sterilize due to heavy infestation with microbes. Therefore, these materials require special treatments prior to use major sterilizing agent (Karataş and Aasim, 2014; Dogan et al., 2016). Surface sterilization of pistachio is also one of the major problem for establishing the plant tissue culture protocol (Mascarello et al., 2007). The surface sterilization protocol of such material is generally based on multisteps like cleaning of explants with water, soaps, shampoos, treating explants with fungicides or bactericide, use of antibiotics and alcohol for a certain period of time (Tilkat and Akdemir, 2013; Benmahioul, 2017; Barpete et al., 2019). Elimination of contaminants in pistachio is very important for the establishment of in vitro regeneration protocol. On the other hand, exudation of phenolic compounds from explants of plants like pistachio also cause serious problem which may lead to death of explants (Leng et al., 2009; Marin et al., 2017). Therefore, optimization of proper sterilization protocol is prerequisite for the establishment of in vitro micropropagation of pistachio. Previous studies on pistachio revealed the application of different sterilizing agents with multisteps of exposing explants to other disinfectants (Benmahioul et al., 2016). The results obtained in this study revealed the significant impact of different internal and external factors like collection time, subculture, sterilizing agent, concentration and sterilization time (Tilkat and Akdemir, 2013).

Collection of plant material is highly significant for successful in vitro sterilization of pistachio. In this study, plant materials collected at different physiological stages resulted in different response towards sterilization. The best time for the collection of plant samples was the month of June followed by May and September. Whereas, collection of plant material in other months responded variably with high in vitro contamination and exedution of phenolic compounds. The impact of season on in vitro contamination of pistachio explants has been documented by Benmahioul et al. (2016) and Benmahioul (2017). The possible reason might be the physiological age and dormancy found in the axillary bud and best time for the collection of plant material is spring or juvenile phase with more rapid cell division and growth (Benmahioul, 2017). High frequency of sterilized explants collected duuring May and June for both rootstocks confirmed the results of Benmahioul et al. (2016).

Presence of *in vitro* contamination and exudation of phenolic compounds in the culture medium are the major limiting factors for successful in vitro regeneration. In order to overcome the issue, selection of proper sterilizing agents, concentration and exposure time are important. Previous studies on pistachio revealed the use of different sterilizing agents like $HgCl_2$, $H_2O_2$ (Ozden-Tokatli et al., 2005) and NaOCl (Kılınç et al., 2015). In all these studies, researchers employed different concentration and treated explants for different time. The results of experiment 2 revealed the supermacy of $HgCl_2$ than other sterilizing agents for inducing high sterilization frequency. The results are in line with the findings of Benmahioul (2017), who reported more contamination on explants treated with NaOCl (84.7%) compared to $HgCl_2$ (38.7%). Whereas, Benmahioul et al. (2016) reported statisitcally insignificant impact of $HgCl_2$ (4.4%) and NaOCl (4.2%) for pistachio.

Application of Huwasan (10%) in this study resulted in 20-30% contamination free explants of UCB-1. Whereas, 100% contamination was recorded for Buttum. Contrarily, successful utilization of 10.0% $H_2O_2$ for surface sterilization of pistachio has been reported by Ozden-Tokatli et al. (2005). Application of 10.0% NaOCl was least effective than other sterilizng agents used in this study and resulted in 100% contamination rate. The previous study on pistachio by Tilkat et al. (2009) did not support the results and they achieved 100 % sterilized eplants of *P. vera* cv. Atli. By exposing explants to 10% (v/v) NaOCl for 30 min. These results clearly revealed the varaiable impact of sterilizing agents and success of sterilization is dependent on genotype used.

Exudation of phenolic compounds in the regeneration medium is the major threat to the in vitro regeneration of most of the plants like pistachio. In order to overcome the issue, incorporation of chemicals like PVP (Aasim et al., 2010), antioxidants like ascorbic acid (Marin et al 2017) or continuous subculture of explants are employed. The results clearly revealed the signfiicant impact of subculture on eliminating or minimizing the exudation of phenolic compounds. The positive impact of subculture on phenolic compounds has been documented for pistachio (Benmahioul, 2017). Application of PVP alongwith BAP also regulated the phenolic compounds exudation. These results suggest that proper combination of PVP+BAP can be useful for inhibiting phenolic compounds with the addition of subculture

## 5. Conclusion

Selection of proper sterilizing agent with concentration and sterilization time regulate the *in vitro* sterilization process for attaining healthy and contamination free explants for *in vitro* regeneration. On the other hand, this study also revealed the significance of sterilizing agent, rootstock type and plant material collection time. Application of 0.1%

$HgCl_2$, material collection during June or May alongwith continuous subculture are optimized for both UCB-1 and Buttum rootstocks. On the other hand, there is also need to perform more experiments to achieve healthy explants with no or minimum releaase of phenolic compounds.

**Conflict of Interest**

Authors have declared no conflict of interest.

**Authors' Contributions**

The authors contributed equally.

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