

Effects of different surface sterilization protocols on fungal load and germination of black henbane seeds

Gursel KARACA¹, Hikmet DEVECİ², Ilknur ALBAYRAK³, Nilgun GOKTURK BAYDAR³

¹Isparta University of Applied Sciences, Agricultural Faculty, Plant Protection Department, 32260, Isparta

²Akdeniz University, Agricultural Faculty, Field Crops Department, 07070, Antalya

³Isparta University of Applied Sciences, Agricultural Faculty, Agricultural Biotechnology Department, 32260, Isparta

Corresponding author: G. Karaca, e-mail: gurselkaraca@isparta.edu.tr

Author(s) e-mail: hikmetdeveci94@hotmail.com, ilknuralbayrakk@outlook.com, nilgunbaydar@isparta.edu.tr

ARTICLE INFO

Received: August 15, 2024

Received in revised form: October 3, 2024

Accepted: October 10, 2024

Keywords:

Hyoscyamus niger
Plant tissue culture
Seed-borne fungi
Sterilization method

ABSTRACT

Black henbane (*Hyoscyamus niger* L.) is an important medicinal plant rich in tropane alkaloids with significant pharmacological effects. To extract these valuable metabolites, seeds are mostly used as the initial material in tissue culture techniques. Due to the intricate surface structure of the seeds, coupled with cultivation of the plant under unsuitable ecological conditions, a high risk of contamination during *in vitro* culture arises. As such, it is important to determine the most suitable sterilization method for successful germination of black henbane seeds under *in vitro* conditions. In this study, the effects of 10 different sterilization protocols with ethyl alcohol (EtOH), sodium hypochlorite (NaOCl), copper sulfate (CuSO₄), hydrogen peroxide (H₂O₂), mercury chloride (HgCl₂), and silver nitrate (AgNO₃), on reduction of the fungal contamination of black henbane seeds was determined by the agar test method. Additionally, the germination rates, as well as shoot lengths and fresh plant weights of the germinated seedlings, were investigated. As a result, it was found that *Alternaria*, *Fusarium*, and *Penicillium* species were the most common fungi on black henbane seeds. Among the chemicals used in the sterilization protocols, AgNO₃ was found as the most effective one, completely inhibiting fungal growth. Sterilization protocols with AgNO₃ also yielded the highest germination rates.

1. Introduction

Plant tissue culture is a technique where new tissues, plants, or plant products can be regenerated from plant cells, tissues, or organs, under aseptic and controlled conditions using artificial nutrient media. In cases where aseptic conditions are not fully ensured in tissue culture environments, microorganisms are able to proliferate and cause contamination of the nutrient media. Microbial contaminations typically occur due to insufficient sterilization of the working environment and equipment or contamination from the explant, posing a significant problem that threatens the development and sustainability of *in vitro* cultures. These microorganisms are competing with *in vitro* cultures by using and rapidly consuming the nutrient medium, causing insufficient growth, tissue necrosis, decreased shoot proliferation and root growth, and ultimately death (Kane 2000; Oyebanji et al. 2009). In addition to the sterilization of the culture medium and equipment, especially surface sterilization of the plant tissues has great importance in preventing the contamination problem that causes significant damage in plant tissue culture (Misra and Misra 2012). Surface sterilization is defined as the process of completely eliminating the microorganisms from the explant without damaging the explant tissue (Sen et al. 2013). For a successful plant tissue culture system, an effective sterilization method must be selected (Oyebanji et al. 2009). Explant-based infections vary according to the vegetation period, environmental conditions stress factors and the plant organ of the explant. Plant tissues and organs, in contact with the soil, generally pose a

higher contamination risk (Battal et al. 2019). Seeds are the plant materials, most frequently used as the initial material for plant tissue culture studies. However, seeds collected from open fields are likely to be highly contaminated with exogenous and endogenous microbial contaminants, including fungi and bacteria. Therefore, seeds, along with tissues and organs in contact with soil are carrying higher risk of contamination than other above-ground explants (Wahyono et al. 2018). The presence of irregularities and protrusions on the seed surface particularly make the surface sterilization of seeds even more challenging (Barampuram et al. 2014).

Fungi are the most important group of seed contaminating microorganisms (Kesho and Abebe 2020). They can contaminate seeds both in the field before harvest and under storage conditions afterward. The most common fungi that contaminate seeds before harvest are; *Alternaria*, *Cladosporium*, and *Fusarium* species, while those found on seeds kept under storage conditions are *Aspergillus*, *Penicillium*, and *Rhizopus* species (Amza 2018). Fungi growing on seeds not only reduce or eliminate the germination capacity of the seeds but also change some physiological and biochemical properties of the seeds (Rao et al. 2014). Elimination of the fungal contamination, which is initially encountered and causes significant losses of plant material, constitutes one of the most crucial stages of an effective tissue culture study. Ethyl alcohol (EtOH), sodium hypochlorite (NaOCl), mercury chloride (HgCl₂), hydrogen peroxide (H₂O₂),

silver nitrate (AgNO₃) and nano silver are among the most preferred chemicals in the surface sterilization process (Abdi et al. 2008). The morphological characteristics and growing conditions of the explant source, type of the explant, and duration and concentration of the chemical used for sterilization are critical factors for successful surface sterilization. Furthermore, the chemical used for sterilization must be economical, have a broad spectrum activity, and be easily removed from the plant material and break down without leaving toxic residues that would prevent cell and tissue development of the explant (Tort 1997).

Nowadays, tissue culture studies are intensively carried out on medicinal plants both for *in vitro* propagation and obtaining valuable plant metabolites with pharmacological effects, and seeds are generally used as initial material. Black henbane (*Hyoscyamus niger* L.), rich in tropane alkaloids such as hyoscyamine and scopolamine with high pharmacological effects, is one of the most important medicinal plants. Although seeds are frequently used as initial materials in the *in vitro* production of the valuable tropane alkaloids contained in the plant, the intricate surface structure of its seeds and the cultivation of the plant under unsuitable ecological conditions without disease and pest control, pose a high risk of contamination during *in vitro* culture (Aljibouri et al. 2012; Ghorbanpour et al. 2013). Therefore, it is important to determine the most suitable sterilization method for the successful germination of black henbane seeds under *in vitro* conditions. In this study, the effects of different sterilization protocols, applied to black henbane seeds, on their fungal load and germination rates, as well as the shoot lengths and fresh plant weights of the germinated seedlings, were investigated.

2. Materials and Methods

2.1. Sterilization and culture of the seeds

Black henbane seeds obtained from Istanbul Zeytinburnu Municipality, Medicinal and Aromatic Plants Directorate were used as plant material. In order to break the dormancy of the black henbane seeds and increase their low germination rate under normal laboratory conditions, the seeds were soaked in 250 mg L⁻¹ gibberellic acid (GA₃) solution for 48 hours, before being transferred to a nutrient media (Ghorbanpour et al. 2013). Then the seeds were sterilized using ten different protocols containing EtOH, NaOCl, CuSO₄, H₂O₂, HgCl₂, and AgNO₃ given below:

1. Soaking in 70% EtOH for 30 seconds + washing with sterile distilled water + shaking in 20% NaOCl solution with 2-3 drops of Tween 20 for 20 minutes + washing thrice with sterile distilled water, each for 5 minutes,
2. Shaking in 10% NaOCl solution with 2-3 drops of Tween 20 for 15 minutes + washing thrice with sterile distilled water + shaking in 5% NaOCl solution with 2-3 drops of Tween 20 for 5 minutes + washing thrice with sterile distilled water, each for 5 minutes,
3. Shaking in 0.05% CuSO₄ solution for 5 minutes + washing thrice with sterile distilled water, each for 5 minutes,
4. Soaking in 70% EtOH for 30 seconds + washing with sterile distilled water + shaking in 0.05% CuSO₄ solution for 5 minutes + washing thrice with sterile distilled water, each for 5 minutes,
5. Washing with detergent + washing thrice with sterile distilled water + shaking in 0.2% HgCl₂ solution for 20 minutes + washing thrice with sterile distilled water, each for 5 minutes,

6. Soaking in 70% EtOH for 30 seconds + washing with sterile distilled water + shaking in 0.1% HgCl₂ solution with 2-3 drops of Tween 20 for 10 minutes + washing thrice with sterile distilled water, each for 5 minutes,

7. Soaking in 70% EtOH for 30 seconds + washing with sterile distilled water + shaking in 20% H₂O₂ (35% stock) solution with 2-3 drops of Tween 20 for 30 minutes + washing thrice with sterile distilled water, each for 5 minutes,

8. Shaking in 20% H₂O₂ (35% stock) solution with 2-3 drops of Tween 20 for 30 minutes + washing thrice with sterile distilled water, each for 5 minutes,

9. Soaking in 70% EtOH for 30 seconds + washing with sterile distilled water + shaking in 1% AgNO₃ solution with 2-3 drops of Tween 20 for 30 minutes + washing thrice with sterile distilled water, each for 5 minutes,

10. Shaking in 1% AgNO₃ solution with 2-3 drops of Tween 20 for 30 minutes + washing thrice with sterile distilled water, each for 5 minutes.

After surface sterilization, the seeds were transferred to Petri dishes containing 30 ml of MS (Murashige and Skoog 1962) nutrient medium supplemented with 30 g L⁻¹ sucrose and 6 g L⁻¹ agar and incubated at 25°C in the dark (Aljibouri et al. 2012). The experiment was set up in 3 replicates, with 5 Petri dishes in each replicate and 10 seeds in each Petri dish.

2.2. Determination of the fungal contamination of the seeds

Black henbane seeds, subjected to 10 different sterilization protocols, were examined for fungal contamination 7 days after being transferred to the nutrient medium. To determine the natural fungal load of the seeds, 100 seeds were placed on the medium without any sterilization treatment, forming the control group. Fungi on the seeds were examined under a stereomicroscope and grouped according to their colony characteristics. Additionally, preparations were made using lactofuchsin and examined under a microscope (Zeiss Axiostar 1061-030). They were identified at the genus level based on morphological characteristics using relevant references (Ellis 1971; Samson et al. 1995; Watanabe 2002). Fungal contamination rates were also determined using the following formula.

$$\text{Contamination rate (\%)} = \frac{\text{Number of infected seeds} \times 100}{\text{Total number of seeds}} \quad [1]$$

2.3. Determination of the germination rates of the seeds and seedling development

To determine the effects of different sterilization protocols on the germination rates of black henbane seeds, sterilized seeds were incubated on the medium at 25°C, in the dark for 15 days and their germination rates were determined using the following formula.

$$\text{Germination rate (\%)} = \frac{\text{Number of germinated seeds} \times 100}{\text{Total number of seeds}} \quad [2]$$

After germination, seedlings were kept at the same temperature but under 16:8 hours light: dark conditions. After one month of growth following seed sowing, the shoot length of the harvested plants was determined using a ruler. The fresh weights of the plants were determined in grams after being weighed on an analytical balance (Kern PLJ 720-3A).

2.4. Evaluation of the results

At the end of the experiment, all data were subjected to analysis of variance using the JMP 17 program, and means were compared by Tukey's multiple comparison test. Variance analysis was performed after applying arc sin transformation to percentage values.

3. Results and Discussion

3.1. Effect of surface sterilization protocols on fungal loads of the seeds

It was determined in the study that fungal growth started on the seeds within 2-3 days of being transferred to the nutrient medium following the sterilization protocols. Microscopic examinations after seven days of incubation revealed that the seeds were mostly contaminated with *Alternaria*, *Fusarium*, and *Penicillium* species. These three genera are among the most common seed-borne fungi (Amza 2018). Species belonging to the genera *Cladosporium*, *Mucor*, *Rhizopus*, *Stachybotrys*, *Stemphylium*, and *Trichoderma* were determined at lower rates on the seeds. It was determined that the contamination rate was quite high (86%) on the seeds incubated without sterilization and some seeds were contaminated with more than one fungus. *Penicillium* species were the most common fungi (51%) in the control group, while the contamination rates of other fungi were lower (Table 1). No information was found regarding seed-borne fungi in black henbane seeds in literature. Therefore, the fungi found on black henbane seeds in this study represent the first record in this regard. *Alternaria* species, which are among the most common seed-borne pathogens, have been reported to have negative effects on the physical properties of seeds in addition to causing diseases in plants (Rathod 2012). *Fusarium* species are also considered as important seed-borne plant pathogens, causing serious economic losses by causing diseases such as root rot and wilt on plants grown from contaminated seeds (Blanco and Aveling 2018). Although *Penicillium* species are common on seeds, they are considered as saprobes causing no disease in developing plants (Kaygusuz and Coşkuntuna 2022). However, contamination of the seeds with these three groups of fungi is undesirable, not only for their negative effects on seed quality and viability but also for their ability to produce mycotoxins that pose a great health concern to humans and animals (Martin et al. 2022).

The sterilization process with CuSO_4 was not effective in reducing the contamination of *Penicillium* species on the seeds, while other chemicals used in the sterilization process completely prevented the growth of this fungus. Similarly, the use of CuSO_4 (protocol no 3) and EtOH and NaOCl (protocol no 1) were not sufficient to reduce the development of *Fusarium* species on the seeds, whereas other applications significantly suppressed the development of the fungus (Figure 1). *Alternaria* species were completely inhibited in applications containing AgNO_3 (Table 1). Considering the total fungal load on the seeds, the AgNO_3 application, which completely eliminated fungal contamination, was determined to be the most effective sterilization protocol, while the effects of EtOH + AgNO_3 , EtOH + H_2O_2 , and H_2O_2 applications were also statistically in the same group (Figure 2). In a similar study, where AgNO_3 yielded positive results, it was found that soaking plum (*Prunus domestica* L.) shoots in 1% AgNO_3 solution for 20 minutes resulted in 96.67% healthy and 3.3% contaminated buds (Ugur 2020). Nartop (2019), investigating the effects of silver nanoparticles on the sterilization efficiency and germination rates of seeds of different plant species, demonstrated that silver nanoparticles were more effective than NaOCl. Conflicting results were obtained with H_2O_2 . Barampuram et al. (2014) reported that H_2O_2 usage in the surface sterilization stage of cotton (*Gossypium hirsutum* L.) seeds resulted in very low levels of fungal contamination. However, in another study, it was found that surface sterilization of peach (*Prunus persica* L.) Batsch shoots with H_2O_2 caused high contamination (Al Ghasheem et al. 2018).

It was observed in the study that the sterilization protocols performed with NaOCl, CuSO_4 , and HgCl_2 were not sufficiently effective, although they slightly reduced the fungal contamination on the seeds by inhibiting the development of certain fungi such as *Penicillium* species. There are different findings regarding the effects of NaOCl application. Oyebanji et al. (2009) conducted a study with cowpea (*Vigna unguiculata* L.) Walp.), rice (*Oryza sativa* L.), and sorghum (*Sorghum bicolor* L.) Moench) seeds, where seeds were sterilized using three different methods involving various durations of EtOH, NaOCl, and their sequential use. As a result, they found that treating the seeds with 3.5% NaOCl for 20-45 minutes was the most effective method for preventing fungal contamination. In another study, NaOCl at 5% concentration for 5 minutes caused

Table 1. The effects of different surface sterilization protocols on the fungal contamination rates of black henbane seeds

Protocol number	Total contamination*	<i>Alternaria</i> spp.	<i>Fusarium</i> spp.	<i>Penicillium</i> spp.	Other fungi
1	40.00 b**	28.00 abcd	12.00 ab	0.00 b	0.00 c
2	56.00 ab	52.00 a	4.00 b	0.00 b	0.00 c
3	74.00 a	20.00 abcd	14.00 ab	38.00 a	12.00 b
4	30.00 b	28.00 ab	2.00 b	0.00 b	0.00 c
5	36.00 b	32.00 abc	4.00 b	0.00 b	0.00 c
6	26.00 bc	26.00 abc	0.00 b	0.00 b	0.00 c
7	6.00 d	6.00 bcd	0.00 b	0.00 b	0.00 c
8	6.00 cd	2.00 cd	4.00 b	0.00 b	0.00 c
9	2.00 d	0.00 d	2.00 b	0.00 b	0.00 c
10	0.00 d	0.00 d	0.00 b	0.00 b	0.00 c
Control	86.00 a	29.00 ab	37.00 a	51.00 a	37.00 a

*Statistical analysis was performed after applying arc sin transformation to percentage values, but actual values were given in the table. **Means on the same column shown with the same letter are not statistically different from each other according to Tukey's test ($P \leq 0.05$).

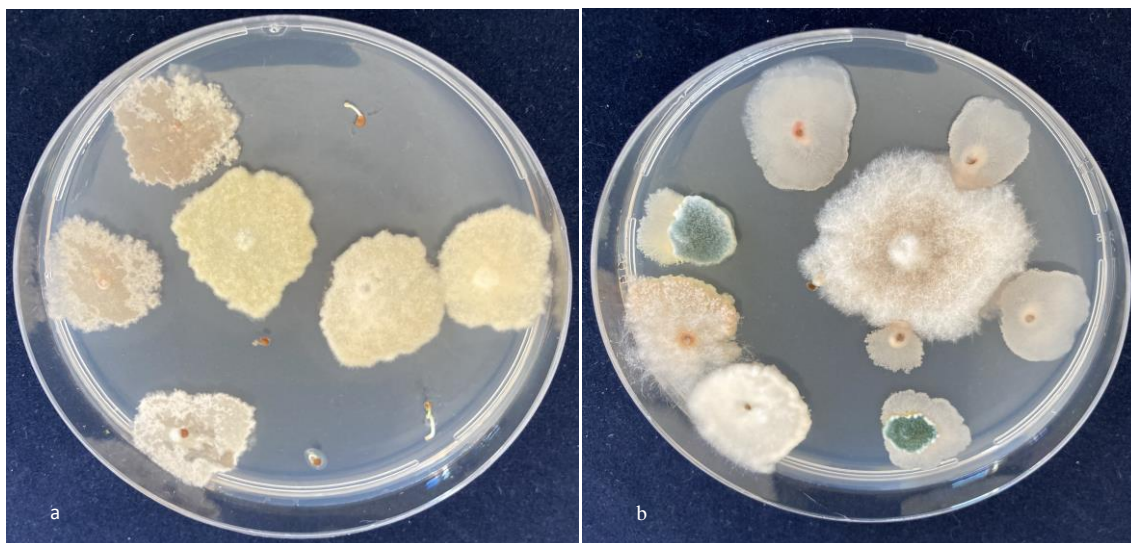


Figure 1. Fungal contamination on the henbane seeds surface sterilized with EtOH + 20% NaOCl (a) and 0.05% CuSO₄ (b).

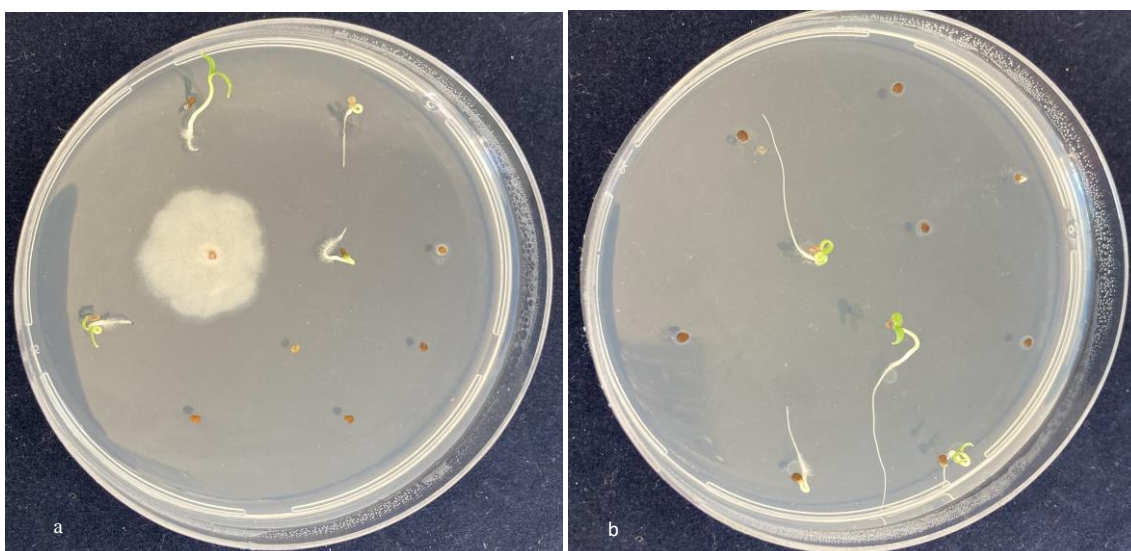


Figure 2. Effects of sterilization protocols with 20% H₂O₂ (a) and 1% AgNO₃ (b) on the fungal contamination of black henbane seeds.

100% fungal contamination after a 7-day incubation period and it was concluded that NaOCl was more effective against bacteria than fungi (Pinto et al. 2012). CuSO₄ has been used to eliminate microbial contamination on seeds since ancient times (Rai et al. 2018). It was found that CuSO₄ treatment of lettuce (*Lactuca sativa* L.) seeds significantly reduced bacterial leaf spot contamination without causing phytotoxicity (Carisse et al. 2000). A study using HgCl₂ in the sterilization of *Catharanthus roseus* (L.) G. Don seeds resulted in high contamination (Ramandi et al. 2019).

3.2. Effects of surface sterilization protocols on seed germination and seedling development

It was determined that the different sterilization protocols caused statistically significant differences in the germination rates of the black henbane seeds and the shoot lengths and fresh weights of the seedlings (Table 2). The intense contamination resulting from the applications of NaOCl, CuSO₄, and H₂O₂ used in surface sterilization also negatively affected the germination

of seeds. Treating plant seeds with NaOCl can affect the germination process by causing changes in seed metabolism. NaOCl has been reported to either promote, inhibit, or have no effect on seed germination in different species depending on the concentrations (Ditomaso and Nurse 2004; Shabana et al., 2021). On the other hand, CuSO₄ added to the *in vitro* nutrient medium containing *Piper nigrum* L. shoots significantly increased shoot development with minimal contamination, indicating that the effectiveness of CuSO₄ in sterilization may also vary depending on the source of the explant used (Rajmohan et al. 2010). A similar situation was observed for the H₂O₂ applications. In this study, the treatments using H₂O₂ caused complete contamination of the black henbane seeds, thereby preventing their germination. Al Ghasheem et al. (2018) determined that surface sterilization of *P. persica* shoots with H₂O₂ negatively affected plant development by damaging explant tissues. However, Barampuram et al. (2014) reported that the use of H₂O₂ in the surface sterilization of cotton seeds had no significant effect on seed germination. The lowest germination rate of 11.79% was found in the seeds sterilized using protocol

Table 2. Effects of different sterilization protocols on seed germination rates of black henbane seeds and, shoot lengths and fresh weights of the seedlings

Protocol number	Germination rate (%)	Shoot length (cm)	Seedling fresh weight (g)
1	-	-	-
2	-	-	-
3	-	-	-
4	-	-	-
5	11.79 c*	5.42 c	0.13 c
6	64.50 b	7.14 b	0.37 b
7	-	-	-
8	-	-	-
9	82.45 a	7.05 b	0.35 b
10	79.27 a	7.44 a	0.41 a

* Means on the same column shown with the same letter are not statistically different from each other according to Tukey's test ($P \leq 0.05$).

number 5 (washing with detergent + distilled water + shaking in 0.2% HgCl₂ solution for 20 minutes + rinsing thrice with sterile distilled water, each for 5 minutes). In protocol number 6, HgCl₂ was used at a lower concentration, and for a shorter duration, the germination rates of the seeds were significantly higher (64.50%). These results indicate that the use of HgCl₂ at a lower concentration for a shorter application time, following ethyl alcohol treatment caused less damage to the seeds. In a similar study, researchers found that HgCl₂ used for surface sterilization of *C. roseus* seeds reduced germination rates due to damage to the seed surface (Ramandi et al. 2019). Researchers investigating the effects of HgCl₂ on surface sterilization of sugarcane buds found that 55% of the explants died after two weeks of incubation, indicating that HgCl₂ was phytotoxic to plant tissues (Danso et al. 2011).

In the present study, the highest germination rates, the highest values for both shoot length, and seedling weight were obtained with protocol number 10, where sterilization was performed by soaking in a 1% AgNO₃ solution for 30 minutes. These results showed that AgNO₃ had lesser negative effects on plant development compared to other applications. Similarly, it was reported that the shoot lengths of *P. domestica* explants sterilized with AgNO₃ were longer (Ugur 2020). Results of another study showed that the effects of AgNO₃ on the germination rates of the seeds varied depending on the plant species (Nartop 2019).

4. Conclusion

This study aimed to determine the most effective surface sterilization method for the successful germination of black henbane seeds under *in vitro* conditions. For this purpose, 10 different sterilization methods using various combinations of EtOH, NaOCl, CuSO₄, H₂O₂, HgCl₂, and AgNO₃ were tested. It was found that 1% AgNO₃ application to the seeds totally inhibited fungal growth without causing phytotoxicity. However, various studies showed that the effectiveness of chemicals used for surface sterilization varied depending on many different parameters such as; the type of explant, source of the explant, plant species and age, preferred chemical, application time, and concentration. Therefore, these factors should be considered when selecting the chemical to be used for the sterilization of any plant material.

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