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The Influence of Disinfection Method on the Contamination Rate of Explants from Ornamental Grasses *Carex montana* and *Sesleria caerulea, In vitro*

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ABSTRACT

Sterilizing underground parts such as rhizomes and roots is a challenging step in tissue culture and a complication faced by most researchers. Therefore, this research aimed to develop a protocol for achieving this goal. Sterilizing agents such as silver nitrate ($AgNO_3$), silver nanoparticles (NSPS), and activated charcoal were used at different concentrations and durations. It was found that immersing the crowns of two ornamental grass species in 0.5% $AgNO_3$ for 20 minutes increased the percentage of uncontaminated explants. The regenerated shoots were observed to be healthy when cultured on all media types. However, the treatment did not prevent necrosis. On the other hand, immersing *Sesleria caerulea* explants in 0.25% silver nitrate ($AgNO_3$) for 10 or 15 minutes with culture media of silver nanoparticles (NSPS) significantly increased the percentage of healthy regenerated shoots. This protocol failed to eliminate microbial contamination and did not produce healthy regenerated shoots in the *Carex montana* species. This treatment resulted in a notable percentage of necrosis among contamination-free explants.

تأثير طريقة التطهير على نسبة تلوث عينات نوعين من اعشاب الزينة، *Carex montana* و *Sesleria caerulea*.

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الخلاصة

يعد تعقيم الأجزاء تحت الأرضية، كالجذور والريزومات، خطوةً بالغة الصعوبة في زراعة الأنسجة، وتحديًا يواجه معظم الباحثين. لذا، يهدف هذا البحث إلى تطوير بروتوكول لتحقيق هذا الهدف. استُخدمت عوامل تعقيم، مثل نترات الفضة ($AgNO_3$) وجزيئات الفضة النانوية (NSPs) والفحم المنشط، بتراكيز مختلفة ولفترات زمنية متفاوتة. وجد أن غمر تيجان نوعين من الأعشاب الزينة ، *Carex montana* و *Sesleria caerulea* في محلول نترات الفضة بتركيز 0.5% لمدة 20 دقيقة يزيد من نسبة الأجزاء النباتية غير الملوثة والبراعم المتجددة السليمة عند زراعتها في جميع أنواع الأوساط، ولكنه لا يمنع النخر. مع ذلك، أدى غمر أجزاء نبات *Sesleria caerulea* في محلول نترات الفضة بتركيز 0.25% لمدة 10 و15 دقيقة إلى زيادة ملحوظة في نسبة البراعم المتجددة السليمة عند إضافة جزيئات الفضة النانوية (NSPs) إلى وسط الزراعة. لم ينجح هذا البروتوكول في القضاء على التلوث الميكروبي، ولم ينتج عنه براعم متجددة صحية في نبات *Carex montana*. ومع ذلك، فقد أدى إلى نسبة ملحوظة من النخر بين الأجزاء النباتية الخالية من التلوث.

الكلمات المفتاحية: زراعة الأنسجة، نترات الفضة، الفحم المنشط، تعقيم الأجزاء المستأصلة.

INTRODUCTION

The plant tissue culture technique is commercially significant and plays a vital role in fundamental research areas such as cell biology, genetics, biochemistry, and biotechnology, highlighting its numerous benefits. Additionally, tissue culture enables the production of contamination-free and genetically modified plants. (Murashiga 1974; Gamborg. 2002; Khosroushahi *et al*., 2006; Kazemzadeh *et al*., 2024). The most common problem in the first stage of tissue culture initiation is contamination by microorganisms that can halt plant growth and development. (Oyebanji *et al*., 2009). Some chemicals, materials, and methods are available to control in vitro contamination, but some may have limited effectiveness or be too toxic (Rostami and Shahsavar 2012).

While there are several methods to reduce the risk of bacterial and fungal contamination during in vitro propagation, including meristem culture, which utilizes the contamination-free part of the plant, and repetitive subcultures, there remains a need for a more effective strategy to sterilize plant tissues. This is essential to avoid the labor-intensive trial and error process and the lengthy decontamination procedures. To eliminate ongoing fungal and bacterial contaminations, antibiotics and antifungal agents can be employed to reduce contamination levels; however, it has been noted that antibiotics are typically phytotoxic and can negatively impact multiplication, callus induction, regeneration, and the survival of explants. (Leifert *et al*., 1992; Teixeirada *et al*., 2003).

The chemical materials used for plant explant disinfection most often are sodium hypochlorite (NaClO) or calcium hypochlorite (CaClO), silver nitrate ($AgNO_3$),

antibiotics, and alcoholic solutions (Pindy and Singh, 2012; Cedo *et al*., 2012; Toniga *et al*., 2015).

Those chemical materials as we mentioned previously may lead to irreversible damage to explants, especially when used at inappropriate concentrations (Tymoszuk 2014). However, currently, nanomaterials, particularly nano-silver particles (NSPs), are frequently utilized as antimicrobial agents in both medical and environmental applications. (Mahna *et al*., 2013). Researchers reported that nano-silver particles (NSPs) are useful in removing contamination from explants, whether they have been supplemented to media or the explants were submerged in it. Results Parzymies *et al* (2018) on Pennisetum alopecuroides, Ismael *et al*. (2017) on *Carex muskingumensis*, (Phuong *et al*., 2024) on *Gerbera jamessoni*, (Sarmast *et al*., 2011) on *Areucaria excelsa*, (Tymoszczuk 2014) on Chrysanthemum × grandiflorum showed that using Nano-silver particles reduced the level of contamination. Data from Ismael *et al*., (2017) showed that the most effective method of decontaminating growing *Carex muskingumensis* buds was to soak explants for 10 min in silver nitrate (AgNO3) at a concentration of 2.5 g · dm-3 and then dip them in a 100 mg · dm-3 streptomycin solution.

Antibiotics are used in controlling internal bacterial contaminations and using them may adversely affect the growth and response of explants, and also may induce resistance in bacteria, but generally, they are not suggested for using it in plant tissue culture in vitro (Dabai *et al*., 2007). However, many studies confirmed that antibiotics can cause the incidence of antibiotic-resistant pathogens through induced mutations or by preparing the ground for natural selection, which at last leads to the requirement of new antibiotics (Taji *et al*., 1993). Activated charcoal AC derived from wood has a large internal surface area. When used in nutrient media, it tends to adsorb moderately polar organic compounds more effectively than apolar or highly polar ones. Additionally, activated charcoal shows a greater adsorption preference for aromatic compounds compared to olefinic unsaturation products.(Yam *et al*., 1990). Activated charcoal can change the pH and create a dark environment similar to soil conditions (Ahmadian *et al*., 2013). The supplementation of activated charcoal to the culture medium may promote or inhibit in vitro growth, depending on the species and tissues used (Pan and Staden. 1998), (Ahmadian *et al*., 2013) and (Olha. 2017) confirmed that AC has a significant role in plant tissue culture.

Various sterilization materials are available that effectively disinfect the external surfaces of explants. However, some parts, such as the nodes close to the soil surface, remain challenging to sterilize. This study aims to address that issue, such as disinfecting the nodes that are growing very close to the soil surface.

MATERIAL AND METHODS

- Plant material and explant preparation

To evaluate the effect of Nano-silver particles (NSPS), AgNO3, and NaClO in sterilizing complicated explants of plants, crowns of two species of ornamental grasses Carex montana and Sesleria caerulea explants were used in the present study. A crown of grasses was washed under running tap water after leaves and roots were removed. The explants of two experiments were surface sterilized in detergent for 20 minutes two times and in 70% ethanol for 10-20 seconds, first experiment's explants were followed by 20 min in 1% (v/v) Naocl and 0.5 % (w/v) AgNO3 (Figure 1), and the second one followed by 10

and or 15 min in 0.25 % (w/v) AgNO3 and subsequently washed three times in sterile, distilled water in a laminar chamber (Figure 2).

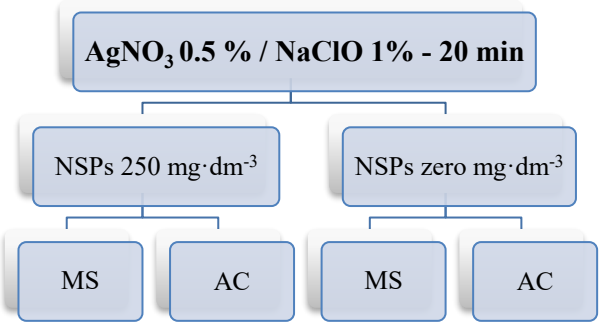

Figure 1. The schema illustrates the pathways for sterilizing and disinfecting two species of ornamental grasses for the first experiment.

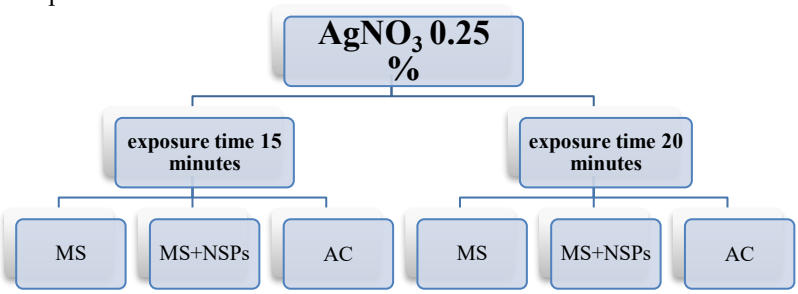

Figure 2. The schema illustrates the pathways for sterilizing and disinfecting two species of ornamental grasses for the second experiment.

- Culture of media.

Three types of media were used in this study for both experiments and prepared according to the MS medium Murashige and Skoog (1962), First basel MS containing salts, organic constituents, and vitamins and supplemented with benzyl adenine (BA) in a concentration of 2.5 mg/L and indole-3-butyric acid IBA in a concentration of 0.1 mg/L , second MS medium was supplemented with 8 mg/L of Nano silver particles solution (Nano-BioTech, particles 6–12 nm) NSPS and third was supplemented with 2 g/L of AC activated charcoal. The pH of the media was adjusted to 5.7-5.8 by 0.1 HCl and NaOH before adding 6.75 g/L of agar to the one liter of media, pouring 8 ml into each tube and autoclaving for 24 minutes at 121 °C/1 bar pressure.

- Isolation and culture conditions in vitro.

First experiment: The sterile crown explants of *Sesleria caerulea* and *Carex Montana* were divided into two combinations. The first combination of explants was soaked in Nano-silver particles and the second one was without soaking and placed into two types of media (MS and AC).

Second experiment: The sterile crown explants of both species of grasses *Sesleria caerulea* and *Carex montana,* were placed in three types of medium (MS, MS+NSPs, and AC).

The tubes were maintained in a growing room in vitro at 22°C during the day and 20°C at night. The light intensity was 30µmol·s-1·m-2 with a 16-hour photoperiod. After 4 weeks, the percentage of contaminated and free of contaminated explants, the percentage of explants with necrosis symptoms, and the percentage of explants that initiated growth (healthy shoots) were recorded.

Design of the experimental and statistical analysis of the data.

The experiments were designed with a Completely Randomized Design (CRD), with twelve replicates for each treatment. Data were statistically analyzed using SAS version 8.2, 2001 (Little *et al*., 2002). The means were compared using Duncan's multiple range tests at a 5% significance level (Al-Rawy & Khalaf Allah, 1980).

RESULTS AND DISCUSSION

In an experiment to disinfect the crown of the ornamental grass plant *Sesleria caerulea*, the results are shown in Table 1. The use of 1% sodium hypochlorite (NaClO 1%) for 20 minutes is ineffective in eliminating microbes and disinfecting explants (0% healthy shoot regenerated), regardless of whether they were soaked for a few seconds in NSPs or not, and regardless of the type of culture medium used (MS with AC or MS without AC). In contrast, the same table showed that treating crown explants of *Sesleria caerulea* with $AgNO_3$ 0.5% were increased the percentage of contamination- free explants (healthy shoots), where regeneration was recorded 32%. Similarly, the same treatment, in which the explants were immersed in nano-silver NSPs for a few seconds and then cultured in a MS medium containing activated charcoal, showed a significant increase in the percentage of healthy shoot regeneration, reaching 26%. Silver is regarded as the second most toxic metal to microorganisms after mercury.

Table 1. Effect of decontamination methods on the rate of contamination-free (healthy regeneration shoots and necrotic), and the rate of contamination (unhealthy regeneration shoots and necrotic), explants of *Sesleria caerulea*. First experiment.

			Free of contamination (%)		Contamination (%)	
Exposure time (min 20)	**NSPs**	**Media**	**Necrosis (%)**	**Regeneration (healthy shoot)(%)**	**Necrosis (%)**	**Regeneration (unhealthy shoot) (%)**
NaClO 1%	0	AC	e 0	e 0	a 73	d 27
		MS	e 0	e 0	b 67	c 33
	250 mg/dm^3	AC	e 0	e 0	b 67	c 33
		MS	e 0	d 3	c 63	b 34
$AgNO_3$ %0.5	0	AC	a 21	c 21	41f	e 17
		MS	a 21	a 32	g 0	a 47
	250 mg/dm^3	AC	c 10	b 26	e 48	f 16
		MS	b 14	c 21	d 58	g 7

The similar letters on values do not differ significantly according to Duncan's multiple test at a 5% probability level.

However, treating the crown explants of Sesleria caerulea with silver nitrate AgNO3 0.5% for 20 minutes, followed by a brief immersion in nano-silver nanoparticles (NSPs), resulted in significant surface sterilization of the explants across all types of MS

media. In contrast, treating the explants with sodium hypochlorite (NaClO 1%) resulted 0% free of contamination.

The same disinfection protocol used in the first experiment was also applied to the second ornamental grass, *Carex montana*, the results of the experiment, as shown in Table 2, indicate that culturing the explants treated with sodium hypochlorite NaClO 1% in an MS medium free of activated charcoal resulted in a notable percentage of contamination-free (regeneration and intact and healthy shoots), which amounted to 5.4 and 5.5, respectively, regardless of whether the explants were briefly immersed in nano-silver particles NSPs or not, in comparison to cultured explants in an MS media that contained activated charcoal AC, where 0% of contamination- free. In general, the use of $AgNO_3$ 0.5% to expose the crown of two types of ornamental grasses, *Sesleria caerulea* and *Carex montana* for 20 minutes, immersing them in nano-silver particles NSPs, and cultured on the MS media with or without activated charcoal AC, resulted in sterile plant parts free of contaminants, in comparison to treating the explants with 1% sodium hypochlorite NaClO which did not achieve the same purpose.

Table 2. Effect of decontamination methods on the rate of contamination-free (healthy regeneration shoots and necrotic), and rate of contamination (unhealthy regeneration shoots and necrotic) explants of *Carex montana*. first experiment

			Free of contamination (%)		Contamination (%)	
Exposure time (min 20)	**NSPs**	**Media**	**Necrosis (%)**	**Regeneration (clean (%) (healthy shoot &**	**Necrosis (%)**	**Regeneration (unhealthy (%) (shoot**
NaClO 1%	0	AC	c 33.33	e 0	f 53.33	b 13.13
		MS	h 0	c 5.4	a 94.59	e 0
	250 mg/dm^3	AC	b 40.74	e 0	g 51.58	c 7.40
		MS	f 11.11	d 5.55	b 83.88	e 0
$AgNO_3$ %0.5	0	AC	g 7.69	e 0	d 76.92	a 15.38
		MS	e 12.12	e 0	c 81.81	d 6.06
	250 mg/dm^3	AC	a 55.17	b 6.89	h 37.93	e 0
		MS	d 25	a 14.28	e 60.71	e 0

The similar letters on values do not differ significantly according to Duncan's multiple test at a 5% probability level.

In the second experiment protocol, an analysis of variance is presented in Tables 3 and 4, evaluating the effect of the previously mentioned treatments on disinfection for two cultivars of ornamental grass. Table 3 illustrates the effect of various exposure times and types of culture media. It shows that an exposure time of 10 minutes did not affect the *Sesleria* c explants and resulted in 0.0% of contamination-free explants, no matter which medium they were cultured in. Explants of the *Sesleria caerulea* plant immersed in $AgNO_3$ 0.5% for 15 minutes and then cultured on MS + NSPs medium effectively controlled fungal and bacterial contamination. The highest significant percentage of healthy regeneration (56%) and 0% necrosis were recorded (Table 3). Simultaneously, the same exposure period for explants, then cultured on MS medium alone also showed a positive effect. Where 45% of healthy regeneration and 0% of necrotic, while the addition of activated charcoal to the media did not show any positive results.

Table 3. Effect of decontamination methods on the rate of contamination-free (healthy regeneration shoots and necrotic), and the rate of contamination (unhealthy regeneration shoots and necrotic)explants of *Sesleria caerulea*. Second experiment.

Exposure time AgNO3 (min)	Media	free of contamination (%)		Contamination (%)	
		Necrosis (%)	Regeneration (healthy (%) (shoot	Necrosis (%)	Regeneration (unhealthy (%)(shoot
10	AC	b 33	d 8	a 0	b 59
	MS+NSPS	a 36	c 9	a 0	c 55
	MS	a 36	c 9	a 0	c 55
15	AC	c 0	e 0	a 0	a 100
	MS+NSPS	c 0	a 56	a 0	e 44
	MS	c 0	b 45	a 0	d 46

The similar letters on values do not differ significantly according to Duncan's multiple test at a 5% probability level..

Table 4 shows the influence of exposure times of $AgNO_3$, 10 and 15 minutes, and types of culture media on the crowns of *Carex montana*. In all treatments, no healthy shoot regeneration was observed, indicating a significant negative effect of the sterilization conditions on explant viability.

10-minute exposure to $AgNO_3$, and placed in the MS+NSPS medium showed the highest percentage of contamination-free explants that showed necrosis (21.42%), compared to the AC medium (9.09%) and the MS medium (0%). Still, contamination levels remained quite high, particularly in the MS medium (81.8%), followed by AC (72.7%). Unhealthy shoot regeneration occurred at low recurrences, with the highest percentage recorded in the MS+NSPS medium (28.57%).

Table 4. Effect of decontamination methods on the rate of contamination-free (healthy regeneration shoots and necrotic), and the rate of contamination (unhealthy regeneration shoots and necrotic)explants of *Carex montana*. Second experiment.

Exposure time AgNO3 (min)	Media	free of contamination (%)		Contamination (%)	
		Necrosis (%)	Regeneration (healthy shoot)(%)	Necrosis (%)	Regeneration (unhealthy (%)(shoot
10	AC	c 9.09	a 0	d 72.7	b 18.18
	MS+NSPS	a 21.42	a 0	e 50	a 28.57
	MS	d 0	a 0	c 81.8	b 18.18
15	AC	b 10.00	a 0	b 90	c 0
	MS+NSPS	d 0	a 0	c 81.8	b 18.18
	MS	d 0	a 0	a 100	c 0

The similar letters on values do not differ significantly according to Duncan's multiple test at a 5% probability level.

When the $AgNO_3$ exposure time was increased to 15 minutes, there was a significant rise in necrosis across all media. The highest contamination rate was noted in the MS medium (100%), and no regeneration, whether healthy or unhealthy, was observed in the MS medium at this exposure time, indicating severe phytotoxic impacts from prolonged $AgNO_3$ treatment.

However, a shorter exposure time (10 minutes) combined with the MS+NSPS medium provided comparatively better decontamination efficiency, although necrosis was still considerable and regeneration remained limited. Prolonged exposure to $AgNO_3$ (15 minutes) had a detrimental effect on explant survival and regeneration, highlighting the importance of optimizing sterilization duration to balance contamination control with tissue viability.

Sodium hypochlorite is one of the most commonly used sterilizing agents in plant tissue culture. However, in this study, a 1% NaClO solution applied for 20 minutes was ineffective at eliminating contamination from crown explants of both species. These results differ from those reported in the scientific literature. e.g, treating the crown of *Sesleria caerulea* with a 1% NaClO for 20 minutes did not prevent the appearance of microbial contamination (Abdi, G., Salehi, H. & Khosh-Khui, M. 2008; Teixeira da Silva *et al*. 2015). Explants below the soil surface are growing in surrounding microbial contamination and may require higher concentrations and longer treatment times. This result is consistent with Bach and Sochacki 2013. and Helem *et al*., 2019.

Our study showed that silver nitrate was found to be more effective in reducing contamination compared to other methods. Silver ions (Ag^+) possess broad-spectrum antimicrobial properties. They achieve this by binding to sulfhydryl groups in proteins, disrupting cell membranes, and interfering with the DNA replication and enzymatic systems of microorganisms. These actions inhibit microbial metabolism, ultimately leading to cell death. However, several studies have reported conflicting observations regarding the growth of treated plants Salachna *et al*., 2019. These results agreed with Teixeira da Silva *et al*. (2016), who used AgNO3 to disinfect saffron corms (Crocus sativus). Parzymies *et al*. (2019) observed that immersing explants and supplementing the medium with NSPs reduced contamination rates in buds and nodal explants of *Pennisetum alopecuroides*.

In the presented research, the use of silver nanoparticles NSPs in the media with immersion of the explants in AgNO3 reduced the contamination rate of explants and significantly increased the healthy shoots capacity. Many researchers have noted a similar effect. Those results were confirmed by Gammoudi *et al*., (2022). and Mihaljevic *et al*., (2013) when they used AgNO3 to eliminate microbial contaminants from explants in in vitro tissue culture.

Research has demonstrated that the supplementation of 50 to 200 mg·dm−3 of silver nanoparticles (NSPs to the growth medium eliminated contamination in cultures of Vanilla planifolia. In the experiment presented, the medium was supplemented with NSPs, resulting in a noticeable reduction in the contamination rate. This finding supports the results reported by other researchers, Spinoso-Castillo *et al*., 2017. Cuba- Díaz*et al*., 2020 observed that treating nodal tissue of *Juncus bufonius* with AgNO3 at 10 mg mL−1 for 10 minutes reduced microbial growth;

One of the most notable findings of this study is the significant difference in response between *Sesleria caerulea* and *Carex montana.* While *Sesleria caerulea* showed moderate regeneration after $AgNO_3$ treatment, *Carex montana* exhibited very limited regeneration and high levels of necrosis under all tested conditions.

Species-specific differences in response to sterilization treatments are common in plant tissue culture. These differences may arise from several biological factors. Varying sensitivity to silver toxicity can influence explant survival. Although silver compounds are

effective antimicrobials, they can also cause phytotoxic effects at high concentrations or with prolonged exposure. Silver ions can interfere with cellular metabolism, alter membrane permeability, and generate oxidative stress by producing reactive oxygen species (Alfosea-Simón*et al*., 2025; Singh *et al*., 2018). Additionally, anatomical differences in crown tissues may impact disinfectant penetration and microbial survival. Dense tissues or lignified structures can hinder the penetration of sterilizing agents, allowing microorganisms to survive within internal tissues. Also, many grass species contain endophytic microorganisms that reside within vascular tissues and cannot be removed by surface sterilization. Endophytic bacterial communities are increasingly recognized as a major source of persistent contamination in plant tissue culture systems (Compant *et al*., 2021; Divya Shree*et al*., 2024).

CONCLUSION

The effects of sterilizing agents, their concentrations, immersion times, and the species of grass varied in the results obtained. The best results were obtained in the first protocol when the explants of *Sesleria caerulea* were immersed in silver nitrate 0.5% for 20 minutes and cultured in a medium containing activated charcoal AC and silver nanoparticles. In the second protocol, the same species performed better when explants were immersed in silver nitrate 0.25% for 15 minutes and cultured in a medium with or without silver nanoparticles. However, obtaining microorganisms free from underground explant contamination is a significant challenge because some microorganisms may develop during the initiation phase of tissue culture and may remain dormant within the tissue cells, only appearing in later stages of culture. Supplementing with activated charcoal AC to the media did not effectively eliminate contamination in two species of ornamental grass, for both protocols used.

CONFLICT OF INTEREST

The authors declare no conflicts of interest associated with this manuscript.

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