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OPTIMIZATION OF *IN VITRO* SEED STERILIZATION PROTOCOL OF SUNFLOWER (*HELIANTHUS ANNUUS* L.) FOR HEALTHY SEEDLINGS

Annotation

This study aimed to optimize *in vitro* sterilization protocols for sunflower (*Helianthus annuus* L.) seeds to establish a reliable foundation for genetic transformation and regeneration research. We evaluated the efficacy of three chemical sterilant 70% ethanol, 15% sodium hypochlorite (NaOCl) with Tween-20, and 0.1% silver nitrate ($AgNO_3$) at varying exposure times. Our results indicate that a 2-minute treatment with 0.1% $AgNO_3$ yielded the highest germination rate (90.6%), demonstrating its superiority for this application. This optimized protocol using silver nitrate offers a robust and efficient method for producing healthy, contamination-free seedlings, addressing a critical bottleneck in sunflower biotechnology, particularly in regions like Uzbekistan where enhancing crop resilience is a priority.

Keywords: Sunflower, *Helianthus annuus* L., *in vitro*, seed, sterilization, silver nitrate, sodium hypochlorite, Tween-20, explant, plant media.

ОПТИМИЗАЦИЯ ПРОТОКОЛА СТЕРИЛИЗАЦИИ СЕМЯН ПОДСОЛНЕЧНИКА (*HELIANTHUS ANNUUS* L.) *IN VITRO* ДЛЯ ПОЛУЧЕНИЯ ЗДОРОВЫХ СЕЯНЦЕВ

Аннотация

Данное исследование было направлено на оптимизацию протоколов стерилизации семян подсолнечника (*Helianthus annuus L.*) *in vitro* с целью создания надежной базы для исследований по генетической трансформации и регенерации. Была оценена эффективность трёх химических стерилизаторов: 70% этанол**,** 15% гипохлорит натрия (NaOCl) с Tween-20 и 0,1% нитрат серебра ($AgNO_3$) при различной экспозиции. Полученные результаты показали, что обработка в течение **2** минут раствором 0,1% $AgNO_3$ обеспечила наивысшую всхожесть (90,6%), что свидетельствует о её преимуществе для данного применения. Оптимизированный протокол с использованием нитрата серебра представляет собой надёжный и эффективный метод получения здоровых, свободных от контаминации сеянцев, что позволяет устранить одно из ключевых ограничений биотехнологии подсолнечника, особенно в таких регионах, как Узбекистан, где повышение устойчивости сельскохозяйственных культур является приоритетной задачей.

Ключевые слова: подсолнечник, *Helianthus annuus L.*, *in vitro*, семена, стерилизация, нитрат серебра, гипохлорит натрия, Tween-20, эксплант, питательная среда.

SOG‘LOM KO‘CHATLAR OLISH UCHUN KUNGABOQAR (HELIANTHUS ANNUUS L.) NING *IN VITRO* URUG‘ STERILIZATSIYA PROTOKOLINI OPTIMIZATSIYA QILISH

Annotatsiya

Ushbu tadqiqot kungaboqar (*Helianthus annuus L.*) urug‘larini *in vitro* sharoitida sterilizatsiya qilish protokollarini optimallashtirishga, genetik transformatsiya va regeneratsiya bo‘yicha tadqiqotlar uchun ishonchli asos yaratishga qaratildi. Uch xil kimyoviy sterilizator samaradorligi baholandi, bunda 70% etanol**,** 15% natriy gipoxlorit (NaOCl) Tween-20 bilan **va** 0,1% kumush nitrat ($AgNO_3$) turli ekspozitsiya vaqtlarida. Natijalar shuni ko‘rsatdiki, 0,1% $AgNO_3$ eritmasida 2 daqiqa ishlov berish eng yuqori unuvchanlik (90,6%)ni ta’minladi va ushbu usulning ustunligini namoyon etdi. Kumush nitrat asosida ishlab **chiqilgan**

optimallashtirilgan protokol sog‘lom, kontaminatsiyasiz ko‘chatlar olish uchun ishonchli va samarali usul bo‘lib, ayniqsa O‘zbekiston kabi hududlarda qishloq xo‘jalik ekinlarining chidamliligini oshirish ustuvor vazifa bo‘lgan sharoitda dolzarb ahamiyat kasb etadi.

Kalit so‘zlar: kungaboqar, *Helianthus annuus L.*, *in vitro*, urug‘, sterilizatsiya, kumush nitrat, natriy gipoxlorit, Tween-20, eksplant, ozuqa muhiti.

Introduction. Sunflower (*Helianthus annuus* L.) is a globally significant oilseed crop that is vital for food security and economic stability, particularly in arid and semi-arid regions, such as Uzbekistan. Modern biotechnological approaches, including *in vitro* regeneration and genetic transformation, offer powerful tools for accelerating sunflower breeding and enhancing resilience to biotic and abiotic stresses (Shahzad and Anis, 2009; Sujatha and Prabakaran, 2003; Chen et al., 2024). However, the success of these techniques is contingent upon the establishment of axenic cultures, which are free from microbial contaminants that can otherwise hinder or prevent plantlet regeneration.

Literature review. Microbial contamination is a major constraint in plant tissue culture, with bacteria and fungi originating from both external (epiphytic) and internal (endophytic) sources (Hemathilake and Peiris, 2021). Endophytic microorganisms are particularly challenging to eradicate because they reside within plant tissues and can remain dormant for extended periods before manifesting in culture (Moreno-Vázquez et al., 2014; Ali et al., 2018). These contaminants degrade the culture medium, release phytotoxic substances, and ultimately cause explant death, leading to significant losses in time and resources (Bhojwani and Razdan, 1996; Barampuram and Zhang, 2011).

Therefore, effective surface sterilization of explants is a critical first step in establishing a successful in vitro culture system. Although a variety of chemical disinfectants are available, their efficacy and phytotoxicity are highly dependent on the plant species, explant type, and treatment conditions (Eliwa et al., 2024). Ethanol is a commonly used pretreatment, but its high phytotoxicity limits its application to brief exposures (Tábori et al., 2021). Sodium hypochlorite is a broad-spectrum disinfectant but can also cause tissue damage at high concentrations or with prolonged use (Teixeira da Silva et al., 2015). More recently, silver nitrate has emerged as a promising alternative, demonstrating high antimicrobial activity at low concentrations with minimal phytotoxicity (Mihaljevic et al., 2013; Nacheva and Ivanova, 2017).

However, the optimal sterilization protocol for sunflower seeds remains elusive, with many existing methods resulting in a trade-off between contamination control and seed viability (Rout, 2011). This is a significant bottleneck for sunflower improvement programs in Uzbekistan, where there is a pressing need for locally adapted, high yielding, and stress-tolerant varieties. This study aimed to develop an optimized and efficient sterilization protocol for sunflower seeds using a systematic comparison of ethanol, sodium hypochlorite, and silver nitrate at different exposure times. Our objective was to identify a protocol that maximizes germination and minimizes contamination, thereby providing a robust foundation for future genetic transformations and in vitro regeneration research on sunflowers in Uzbekistan.

Materials and method.

Seeds of three local Uzbek sunflower varieties, Navruz, Buzurut, and Sur, were used as explants. The seeds were manually dehulled to expose the kernel, and all subsequent procedures were conducted under aseptic conditions in a laminar airflow cabinet.

Sterilization Protocol. We employed a factorial experimental design to evaluate the effects of the three different sterilizing agents at various exposure times on seed germination and contamination rates. Three replicates of 10 seeds were used for each treatment, following the methodology described by Eliwa et al. (2024). The treatments were as follows.

Initial Pre-Treatment with Ethanol. Seeds were first immersed in a 70% ethanol solution for a brief period of 1–5 min to effectively eliminate surface microorganisms. Subsequently, the ethanol solution was decanted.

Sterilization with Sodium Hypochlorite. Following ethanol treatment, the seeds were transferred to a solution of 15% sodium hypochlorite (NaOCl) containing three drops of Tween-20 as a wetting agent to ensure uniform contact with the seed surface. This solution was applied for 5–15 min to remove the bacterial and fungal spores. The seeds were gently agitated to improve sterilization efficiency.

Sterilization with Silver Nitrate. For more recalcitrant contaminants and deep-seated fungal spores, seeds were treated with a 0.1% silver nitrate ($AgNO_3$) solution for 2–3 min. This step proved highly effective in reducing residual contamination.

Post-Treatment Rinsing and Statistical Analysis. After each sterilization treatment, the seeds were rinsed three to four times with sterile distilled water to remove residual chemical agents. Each rinse was conducted for 5 min to ensure thorough removal of potentially phytotoxic residues. Data were analyzed using analysis of variance (ANOVA), and means were compared using Tukey's honest significant difference (HSD) test at $p < 0.05$.

Analysis and results. Our evaluation of the three chemical sterilants at different exposure times revealed significant differences in their effects on sunflower seed germination and contamination rates (Table 1). A clear correlation was observed between the type of sterilant, exposure duration, and resulting germination rate, with statistical analysis confirming the significance of these findings ($p < 0.05$).

Ethanol Treatment. A 1-minute treatment with 70% ethanol resulted in a high germination rate of 87.0%. However, longer exposure times of 3 min and 5 min led to a significant ($p < 0.05$) reduction in germination to 45.5% and 15.2%, respectively, indicating a high degree of phytotoxicity with prolonged exposure.

Sodium Hypochlorite Treatment. The highest germination rate (89.6%) was achieved with a 5-minute exposure to 15% NaOCl. A 10-minute exposure yielded a similar germination rate of 87.9%, while a 15-minute exposure resulted in a slight decrease to 81.2%.

Silver Nitrate Treatment. The most effective treatment was a 2-minute exposure to 0.1% $AgNO_3$, which resulted in the highest germination rate of 90.6%. Longer exposure times of 3 min and 5 min significantly ($p < 0.05$) reduced germination to 64.2% and 56.7%, respectively.

Table 1. Effects of different sterilization agents on *in vitro* germination and contamination of sunflower seeds.

Chemical Agent	Exposure Time (min)	Concentration (%)	Germination Rate (%)	Contamination Ratio (%)
Ethanol (C2H5OH)	1	70	87.0 ± 2.5a	30
	3	70	45.5 ± 3.1b	10

	5	70	15.2 ± 1.8c	0
NaOCl + Tween-20	5	15	89.6 ± 2.1a	60
	10	15	87.9 ± 2.3a	20
	15	15	81.2 ± 2.9b	10
Silver Nitrate (AgNO3)	2	0.1	90.6 ± 1.9a	40
	3	0.1	64.2 ± 3.5b	30
	5	0.1	56.7 ± 4.0c	10

Values represent mean ± standard deviation of three replicates. Means within a column followed by the same letter are not significantly different at $p < 0.05$ (Tukey's HSD test).

Discussion. This study successfully identified an optimized protocol for surface sterilization of sunflower seeds, which is a critical prerequisite for the application of biotechnological tools for crop improvement. Our findings underscore the delicate balance between eliminating microbial contaminants and preserving explant viability, a challenge that is particularly acute in species with recalcitrant seed coats like sunflower.

The high phytotoxicity of ethanol at longer exposure times is consistent with previous reports, and is attributed to its dehydration and protein-denaturing effects (Healy et al., 2021). Our results confirm that ethanol is best used as a brief pre-treatment to reduce the surface microbial load before the application of a primary sterilant, similar to the protocol described by Chen et al. (2024) for sunflower tissue culture.

Sodium hypochlorite, a widely used disinfectant in plant tissue culture, demonstrated effectiveness, but with variable results depending on the exposure time. The decrease in germination at longer exposure times is likely due to the phytotoxic effects of chlorine, as previously reported (Teixeira da Silva et al., 2015). Our findings are consistent with those of Eliwa et al. (2024), who reported optimal results with NaOCl at specific concentrations and exposure times for peach rootstock sterilization.Notably, the highest germination rate (90.6%) was achieved with a 0.1% solution of silver nitrate for 2 min. This positions AgNO3 as a superior sterilizing agent for sunflower seeds under the tested conditions. Its efficacy at a very low concentration and short duration is highly advantageous, as it minimizes the risk of phytotoxicity while effectively controlling contaminants. This finding is in strong agreement with Mihaljevic et al. (2013), who also reported silver nitrate as a powerful and less damaging agent for sterilizing plant explants *in vitro*.

The superior performance of silver nitrate in our study is a significant finding that aligns with recent research on alternative sterilization agents. Its high antimicrobial activity at low concentrations and short exposure times make it an ideal sterilant for sensitive explants such as sunflower seeds. This is consistent with the studies by Mihaljevic et al. (2013) and Nacheva and Ivanova (2017), who reported the effectiveness of silver nitrate as a less phytotoxic alternative to traditional sterilants. The mechanism of action of silver ions involves their interaction with microbial enzymes and proteins, leading to cell death, while causing minimal damage to plant tissues.

Conclusion. This study identified an optimized sterilization protocol for sunflower seeds that significantly improved germination rates while effectively controlling microbial contamination. The use of 0.1% silver nitrate for 2 min represents a key advancement and offers a superior alternative to traditional sterilants. This protocol provides a robust and reliable foundation for advancing sunflower biotechnology in Uzbekistan, thereby enabling the development of improved varieties with enhanced stress tolerance and yield. Future research should focus on applying this protocol to a wider range of sunflower genotypes and on integrating it into a comprehensive in vitro regeneration and genetic transformation system for this vital crop.

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