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Developing an "in vitro" propagation protocol for *Silphium perfoliatum*

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Abstract
The *Silphium* L. species are indigenous to the fields, prairies, and forests of eastern and central North America. The genus *Silphium* (L.) belongs to the family Asteraceae, order Heliantheae. In Europe*, Silphium perfoliatum* was introduced in the 18th century and was initially utilized as an ornamental plant in gardens and parks in Germany, France, Switzerland, and the United Kingdom. Micropropagation represents an alternative means of propagation that can be employed for the mass multiplication of plants in a relatively shorter time frame. The objective of this study was to develop and optimize a protocol that can be readily implemented in field culture. The study demonstrates that the sterilization of seeds with a $CuSO_4$ solution, followed by their immersion in a phenolic biostimulant solution prior to placement on a simple MS culture medium, yielded the most optimal results in the production of *Silphium perfoliatum* explants.

Keywords: cup plant, micropropagation, sterilization, seeds, seedling

Introduction

Silphium perfoliatum, commonly known as "cup plant", is a perennial species of the Asteraceae family native to North America. This plant exhibits remarkable ecological adaptability and significant resistance to abiotic and biotic stresses, and is used in agriculture, biomass production, and plant breeding [18].

Given its efficacy and capacity for biomass production and a range of applications, cup plant represents a promising path of research and development. It is cultivated for a variety of purposes, including as an ornamental plant, a source of honey, for bioenergy production, as a solid biofuel for combustion, as fodder, and as a source of fibre. *S. perfoliatum* has the potential to be utilized in a number of industries, including construction, pharmaceuticals, agrochemicals and food.

In the first year of growth, *Silphium* exhibits the formation of 12-14 rosette leaves and a multitude of lateral roots. In the second year, the plants begin to produce multiple stems, reaching up to three meters in length and exhibiting four edges. The number of stems produced by the plant increases as it matures; 10–25 stems may develop.

The maturation of the inflorescence of *S. perfoliatum* seeds is an irregular and protracted process, occurring over an extended period of time due to the continuous formation of new flowers. This phenomenon results in the harvest of seeds that are at various stages of development, including those that are ripe, unripe, and sterile.

The establishment of a crop of *S. perfoliatum* may be facilitated by the transplantation of seedlings that have been cultivated in a nursery setting. Although this method requires significant investment in terms of cost and time, it is more efficient than using seeds with variable germination rates. This is due to earlier and more consistent development, uniform soil coverage, and higher yields.

Recent research highlights the potential of this species for biogas production, cultivation on marginal lands, and development of pharmaceuticals and cosmetics [15].

The development of an efficient in vitro culture protocol for *Silphium perfoliatum* is essential to capitalize on these opportunities. In vitro culture methods offer advantages such as rapid propagation, preservation of germplasm, and improved phenotypic characteristics of the species [3].

In vitro cultures are fundamental for rapid and controlled propagation of perennials such as S. perfoliatum. These techniques make it possible to obtain a large number of genetically identical plants, reducing variability and ensuring a consistent quality of plant material [6].

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In vitro genetic conservation offers the possibility of storing genetic diversity in a controlled environment, protected from external conditions. The use of Murashige and Skoog (MS) medium supplemented with growth regulators such as BAP (6-benzylaminopurine) and NAA (naphthaleneacetic acid) has been shown to be effective in stimulating organogenetic regeneration of S. perfoliatum explants [5, 17].

Effective sterilization of explants is critical to obtaining viable in vitro cultures. Various sterilization methods, including the use of sodium hypochlorite, hydrogen peroxide, and ethyl alcohol, have been tested to minimize contamination and maximise germination success [7].

Proper sterilization treatments directly affect the germination rate and growth of S. perfoliatum explants. Apical explants derived from sterilized seeds grown on MS media supplemented with 5 mg/L BAP and 1 mg/L NAA showed a regeneration success rate of over 90%, and 41.7% of these explants produced lateral shoots [11].

The regenerated plants were successfully transferred to rooting media without growth hormones and subsequently acclimated under greenhouse conditions, suggesting that *S. perfoliatum* can be efficiently propagated by in vitro culture [1].

In vitro methods allow the development of genotypes with improved traits, including increased tolerance to drought, salinity and pathogens, which is essential in the context of climate change [9, 14].

Silphium perfoliatum has been identified as a resilient species capable of adapting to degraded soils and contributing to soil quality improvement, with potential for crop diversification and bioenergy production [8].

Due to its ability to rapidly produce biomass and adapt to diverse environmental conditions, *Silphium perfoliatum* is a valuable resource for bioenergy, phytoremediation, and nutrient production. The use of in vitro cultures to improve yields and plant characteristics is essential for its integration into sustainable agriculture and ecological recycling processes [10, 4, 12].

Material and Method

1. Sterilization of whole seeds of *Silphium perfoliatum*

Plant material. The seeds of *Silphium perfoliatum* used in the experiments were collected from the experimental fields of the University of Life Sciences "King Mihai I" from Timisoara. The plants were identified and grouped according to their morphological characteristics of interest: plants with 2 leaves and 4 edges, 3 leaves and 6 edges, and 4 leaves and 8 edges, respectively. The seeds were labeled accordingly to allow correlation of the results with the morphology of the source plants.

Thermal stratification. Before starting the experiments, the seeds were subjected to cold stratification. They were kept at 5°C for 4 weeks to improve germination capacity.

A. Sterilization Process with $CuSO_4$

To develop an optimal sterilization protocol, three experimental versions were tested with copper sulfate ($CuSO_4$) and three others with mercuric chloride ($HgCl_2$):

Version I: Pretreatment with distilled water. Seeds were immersed in sterile distilled water for 24 hours using a magnetic stirrer. After pretreatment, the seeds were subjected to

- V1.1 (control): immersion in sterile distilled water without the use of $HgCl_2$;
- V1.2: treatment with 0.1% $HgCl_2$ for 15 minutes;
- V1.3: treatment with 0.2% $HgCl_2$ for 5 minutes.

Version II: Pre-treatment with $CuSO_4$ 0.75%.

The seeds were treated by immersion in $CuSO_4$ 0.75% for 24 hours with constant shaking. After pretreatment, they were sterilized according to the following conditions:

- V2.1 (control): immersion in sterile distilled water without the use of $HgCl_2$;
- V2.2: treatment with 0.1% $HgCl_2$ for 15 minutes;
- V2.3: treatment with 0.2% $HgCl_2$ for 5 minutes.

Version III: Pre-treatment with $CuSO_4$ 0.5%.

Seeds were treated with $CuSO_4$ 0.5% for 24 h with magnetic stirring. Sterilization was performed under these conditions:

- V3.1 (control): immersion in sterile distilled water without the use of $HgCl_2$;
- V3.2: treatment with 0.1% $HgCl_2$ for 15 minutes;
- V3.3: treatment with 0.2% $HgCl_2$ for 5 minutes.

After chemical treatments, seeds were washed three times with sterile distilled water to remove residues.

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Figure 1. *Silphium perfoliatun* seeds on culture medium

B. Sterilization with $HgCl_2$

The following versions have been tested:

- V I (control): seeds were pretreated with $CuSO_4$ 0.5% and washed, without $HgCl_2$ treatment.
- V II: Pre-treatment with $CuSO_4$ 0.5% and sterilization with $HgCl_2$ 0.1% for 5 min.
- V III: Sterilization with 0.1% HgCl for 10 minutes.
- V IV: Sterilize with 0.1% $HgCl_2$ for 15 minutes.

Inoculation and Culture Medium. Seeds of Cup plant were grown on MS (Murashige & Skoog) medium with the following composition per 1 L: macroelement stock solution: 50 ml, sucrose: 30 g, agar: 6.8 g, pH adjusted to 6.8.

The culture medium was sterilized in an autoclave at 120°C for 20 minutes and then dispensed into sterile glass jars (50 ml per jar) (fig. 1).

2. Germination of *Silphium perfoliatum* seeds

To study the increase in the germination percentage of *Silphium perfoliatum seeds*, experiments were carried out using two different methods: the use of a phenol-based biostimulation solution and another based on the supplementation of the culture medium with gibberellic acid (GA_3).

a.Use of a phenol-based biostimulant solution

A 0.1% biostimulant solution was prepared using a combination of phenols (V1). The final volume of the solution was 150 ml and it was sterilized by filtration using a special PES membrane syringe filter with a pore size of 0.22 µm.

Seeds were subjected to a standardized sterilization protocol that included: immersion in 0.5% $CuSO_4$ 0.5% solution for 24 hours with constant agitation, treatment for 10 minutes in 0.1% $HgCl_2$ solution, rinse three times with sterile distilled water to remove any residual chemicals.

After sterilization, seeds were immersed in the sterilized biostimulant solution for 60 minutes.

Treated seeds were inoculated on Murashige & Skoog (MS) culture medium supplemented with macroelements. The culture medium was adjusted to pH 6 and autoclaved at 120°C for 20 minutes. 200 seeds were used, evenly distributed in sterile glass jars containing 50 ml of culture medium each.

b.Supplementation of culture medium with GA_3 for seed germination of *Silphium perfoliatum.*

In version two of the experiment (V2), Murashige & Skoog (MS) culture medium was supplemented with gibberellic acid (GA_3) to stimulate the germination of *Silphium perfoliatum* seeds.

A stock solution of GA_3 at a concentration of 4 mg/ml was prepared and sterilized by filtration through a special PES membrane filter with a pore size of 0.22 µm. The sterilized solution was added to the sterilized culture medium under aseptic conditions before distribution in sterile vessels.

Seeds were subjected to the same sterilization procedure as previously used: immersion in 0.5% $CuSO_4$ 0.5% solution for 24 h with constant agitation, treatment for 10 min in 0.1% $HgCl_2$ solution and three consecutive rinses with sterile distilled water to remove chemical residues.

Incubation conditions. Seed jars were placed in a growth chamber with controlled parameters: temperature: 25°C, humidity: 60-70%, photoperiod 14 hours light / 10 hours dark, light intensity: 40,000 lux.

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Figure 2. *Silphium pefoliatum* seeds in the growing room

Final protocol for in vitro germination of *Silphium perfoliatum* seeds

After analyzing the experimental data, an optimal protocol for in vitro germination of *Silphium perfoliatum* seeds was established. This protocol includes the following steps: soak seeds in 0.5% $CuSO_4$ 0.5% solution for 24 hours using a magnetic stirrer to ensure uniform treatment. sterilize by immersion in 0.1% $HgCl_2$ solution for 10 minutes. Wash the seeds three times with sterile distilled water to completely remove chemical residues. The seeds were then immersed in the sterile biostimulant solution (0.1% phenol concentration) for 60 minutes.

The seeds treated in this way were inoculated on Murashige & Skoog (MS) medium with macro elements and adjusted to pH 6 according to the protocol described above (fig. 2).

Plant acclimatization. After monitoring and confirming the favorable development of the young plants, they were transferred to the acclimatization stage, a crucial process for the gradual adaptation of the plants from the in vitro environment to the ex vitro conditions. This phase involved several critical steps to ensure the successful transition of plants from in vitro to ex vitro conditions. First the plants were carefully removed from the in vitro containers, and their roots were cleaned of debris from the culture medium by washing with warm water. The plants were transplanted onto a substrate consisting of a mixture of soil and perlite that provided adequate structure for drainage and aeration. Each plant was covered with a transparent pot to reduce the shock of sudden exposure to ex vitro conditions and to create a protective microclimate. The plants were watered every two days with room temperature water. The plants were exposed gradually: they were initially exposed for a few seconds and the duration of exposure was progressively increased over the following days until complete acclimatization.

The plants were acclimated for 29 days, during which time they gradually adapted to the new environmental conditions. The plants showed signs of healthy growth and normal development, confirming the effectiveness of the protocol and the success of the transition from an in vitro to an ex vitro controlled environment (fig. 3).

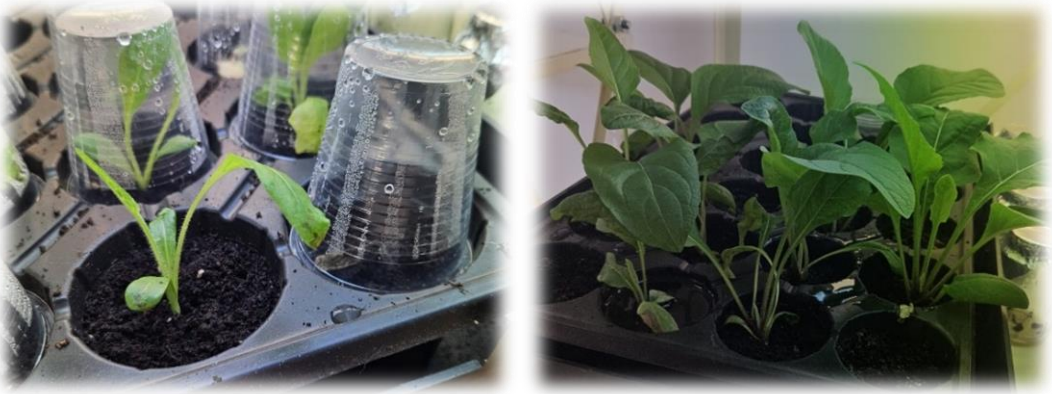

Figure 3. Different stages of acclimation of *Silphium perfoliatum* plants

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Results and Discussion

Sterilization of whole seeds of *Silphium perfoliatum*

1.Version of seed sterilization with $CuSO_4$

The results of the experimental $CuSO_4$ treatments revealed distinct outcomes based on the treatment version. In VI, V1.1, V1.2, and V1.3, all seeds exhibited total infection and no germination occurred.

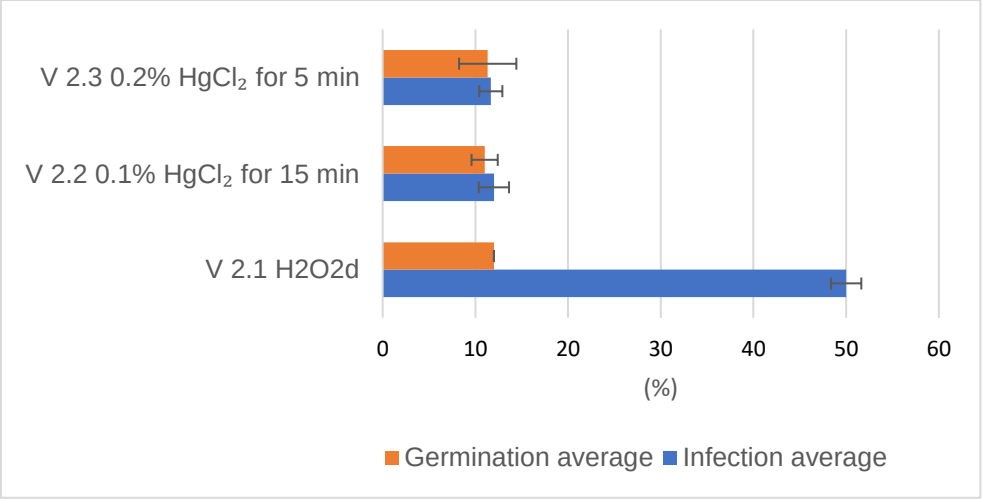

Figure 4. The effect of $CUSO_4$ 0.75% pre-treatment on infection and germination - V II

Treatment with H_2O_2 (V2.1) significantly reduced infection with a mean of 50.00% ± 1.63. In comparison, $HgCl_2$ treatments had a superior effect in reducing infection. For V2.2 ($HgCl_2$ 0.1% for 15 minutes), the mean infection rate was 12.00% ± 1.63, and for V2.3 ($HgCl_2$ 0.2% for 5 minutes), the mean rate was 11.67% ± 1.25. These results suggest a high efficacy of $HgCl_2$ treatments in inhibiting pathogens, with lower variability in the higher concentration and shorter exposure time version (V2.3) (fig.4.).

In terms of germination, the H_2O_2 treatment (V2.1) obtained an average of 12.00% ± 1.41, similar to the values observed for the $HgCl_2$ treatments: 11.00% ± 1.41 for V2.2 and 11.33% ± 3.09 for V2.3. This indicates that although $HgCl_2$ treatments are effective in reducing infection, they may slightly affect germination, especially in the case of the 0.2% $HgCl_2$ treatment for 5 min (V2.3), which had significant variability (σ = 3.09).

The results suggest that $HgCl_2$ treatments are a more effective alternative to H_2O_2 for reducing infection, but the effect on germination must also be considered. Version V2.3 (0.2% $HgCl_2$ 0.2% for 5 min) appears to be the most balanced, with a significant reduction in infection and minimal effect on germination, but the greater variability in germination may indicate the need for further adjustment of treatment conditions.

Effect of pretreating with $CUSO_4$ 0.5% on infection and germination - V III (fig. 5)

The results show that treatment with H_2O_2 (V3.1) maintained a mean infection rate of 31.00% ± 2.45. In comparison, treatments with $HgCl_2$ showed superior efficacy in reducing infection, with mean values of 24.00% ± 1.63 for V3.2 and 23.67% ± 1.70 for V3.3. These results indicate a progressive reduction in infection with increasing $HgCl_2$ concentration and decreasing exposure time.

Treatments with $HgCl_2$ had a positive effect on germination compared to H_2O_{22}. The mean germination for V3.1 was 14.33% ± 2.62, significantly lower than those observed for V3.2 (23.00% ± 1.63) and V3.3 (23.67% ± 0.47). In particular, the V3.3 showed very low variability (σ = 0.47), suggesting a higher consistency of this treatment in stimulating germination.

Analyzing the results for infection and germination together, it can be seen that the treatment with $HgCl_2$ 0.2% for 5 min (V3.3) offers the best combination between reduction of infection (23.67%) and stimulation of germination (23.67%). Also, the low variability of germination for V3.3 indicates a better control of the experimental parameters, making this treatment the most promising from a practical point of view.

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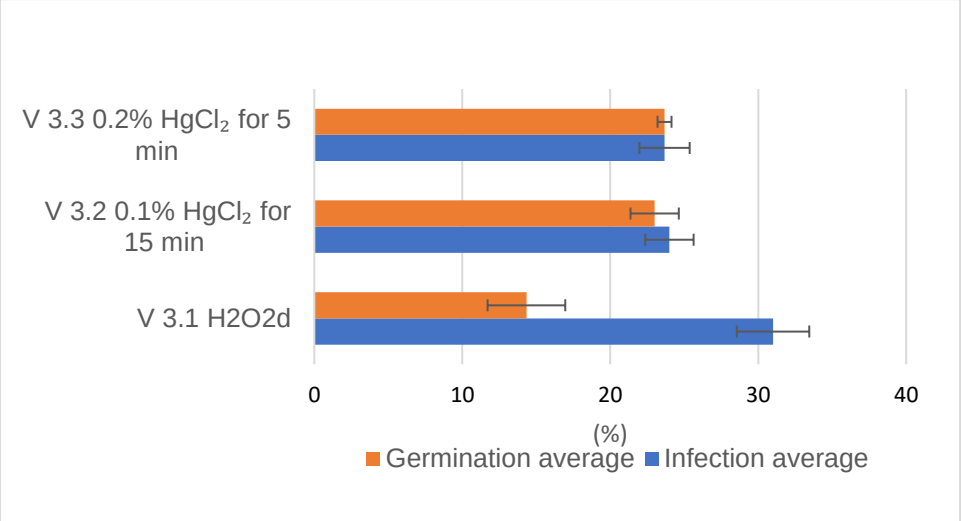

Figure 5. The effect of pretreating with $CUSO_4$ 0.5% on infection and germination - V III

2. Optimization of sterilization with $HgCl_2$

After 41 days of incubation, the results showed variability among the treatment versions based on $HgCl_2$. The H_2O_2 (V1) treatments had an average infection rate of 58.00% ± 4.32, which is the highest value in the experiment. $HgCl_2$ treatments showed higher efficiencies in reducing infection: 27.00% ± 2.16 for V2, 16.67% ± 1.25 for V3 and 15.67% ± 1.70 for V4. These results suggest that longer exposure to $HgCl_2$ contributes to a more significant reduction in infection.

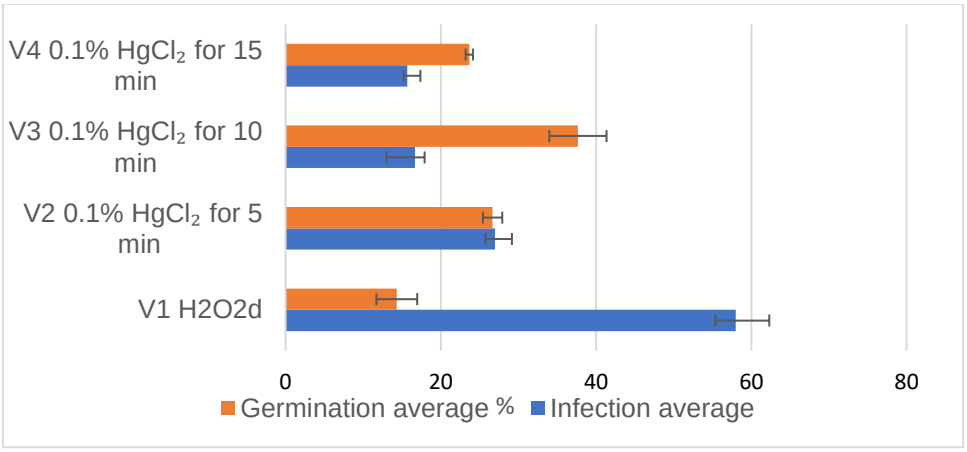

Figure 6. Optimization of sterilization with $HgCl_2$

$HgCl_2$ treatments had different effects on germination. V1 (H_2O_2) treatments had an average germination of 14.33% ± 2.62, which was significantly lower than $HgCl_2$-based treatments. V2 ($HgCl_2$ 0.1% for 5 min) treatments had an average germination of 26.67% ± 1.25, while V3 ($HgCl_2$ 0.1% for 10 min) treatments reached a maximum value of 37.67% ± 3.68. However, for V4 ($HgCl_2$ 0.1% for 15 min), germination decreased to 23.67% ±0.47, suggesting a possible negative effect of prolonged exposure time on the germination process.

The results suggest that treatment with 0.1% $HgCl_2$ for 10 min (V3) is optimal for reducing infection and maximizing germination, with a significant reduction in infection (16.67%) and the highest germination rate (37.67%). The relatively low variability of the data for this treatment also indicates good control of the experimental parameters. However, a longer exposure time (V4) seems to have a negative effect on germination, despite a further reduction in infection.

Increasing the germination percentage of *Silphium perfoliatum* seeds

The results showed that the treatment with phenolic biostimulants (0.1% solution) resulted in an average germination rate of 80.33% ± 1.70, significantly higher than the GA_3 treatment, which recorded an

average germination rate of 65.67% ± 2.05 (fig.7). This indicates a higher efficiency of phenol-based biostimulants in stimulating seed germination under the experimental conditions tested.

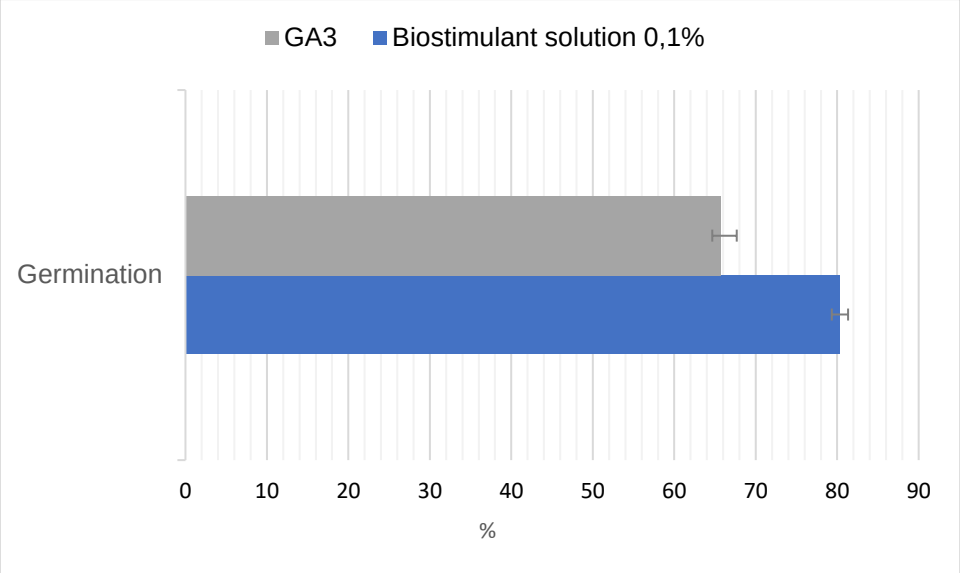

Figure 7. Effect of phenol and GA_3 biostimulant treatments on seed germination of *Silphium perfoliatum* seeds

Treatment with phenolic biostimulants also showed a lower variability ± 1.70 compared to GA_3 treatment (σ = 2.05), indicating a more consistent effect on germination.

These results suggest that phenol-based biostimulants not only improve the germination rate of Cup plant seeds, but also provide greater consistency of effect. This can be explained by the presence of phenolic compounds that play a key role in stimulating physiological processes related to germination, including protection against oxidative stress and stimulation of cell metabolism. In contrast, GA_3 treatment, although effective, produced a lower germination rate and higher variability, suggesting a less robust effect.

Our results are in line with similar research worldwide on the in vitro propagation of *Silphium perfoliatum* species, highlighting the importance of developing efficient protocols for the propagation of this plant with significant agronomic and environmental potential. Tomaszewska-Sowa and Figas [2], developed a micropropagation protocol for *Silphium perfoliatum* using apical explants of seedlings in in vitro culture. Their study emphasized the importance of optimizing sterilization processes and the composition of the culture medium to obtain uniform and high quality plant material.

In another research, Sumalan et al. [13], evaluated the potential of *Silphium perfoliatum* species in bioremediation of heavy metal contaminated soils. Although the focus of the study was on the phytoremediation capacity, the results also emphasized the importance of effective propagation of this species for its application in ecological practices. Ullah et al. [16], investigated the use of *Silphium perfoliatum* as a promising plant for phytoremediation of heavy metal contaminated soils. The study highlighted the need for effective propagation methods for large-scale implementation of this species in ecological remediation programs.

Țîței et al. [19] analyzed the agrobiological peculiarities of *Silphium perfoliatum* species under the conditions of the Republic of Moldova, highlighting its potential as a fodder and energy plant. The study emphasized the importance of developing efficient propagation protocols for the optimal utilization of this species under different agro-ecological conditions.

In addition, recent research by Clapa et al. [3] has demonstrated the effectiveness of using biostimulants in the in vitro propagation of perennial herbaceous species, highlighting the importance of these substances in improving the regeneration rate and quality of the plants obtained.

These studies confirm the validity of our approach in developing an efficient protocol for in vitro germination and propagation of *Silphium perfoliatum* seeds and highlight the importance of optimizing sterilization processes, use of biostimulants, and acclimation steps for successful propagation of this valuable species.

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Conclusions

The main objective of the present study was to develop an efficient protocol for the germination and in vitro propagation of *Silphium perfoliatum* seeds, a species of interest for its agronomic and ecological potential.

The experimental results led to the establishment of a standardized protocol that demonstrated its efficiency in preventing contamination and increasing the germination percentage of seeds. Pretreatment with $CuSO_4$ 0.5% for 24 hours, followed by sterilization with $HgCl_2$ 0.1% for 10 minutes and subsequent immersion in a phenol-based biostimulation solution proved to be an optimal combination for obtaining reproducible results.

The effectiveness of the protocol is demonstrated by the high germination rates and the significant reduction in infections, especially for the seeds treated with the biostimulant solution. In comparison, the use of the GA_3 enriched medium showed moderate benefits in reducing infections, but had a lower germination rate, highlighting the differential role of biostimulants on seed viability.

The final stage of the experiment, plant acclimatization, confirmed the viability of the developed methodology, demonstrating a successful adaptation of young plants to ex vitro conditions in only 29 days. This success validates the proposed protocol as robust, reproducible and applicable to the propagation of *Silphium perfoliatum*.

The results provide important contributions to the in vitro propagation technology of *Silphium perfoliatum*, highlighting key factors such as sterilization, germination stimulation and plant acclimatization. These findings open new possibilities for the implementation of efficient methods for the conservation and utilization of this valuable species in agricultural and ecological contexts.

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