

In Vitro Sterilization Procedures For Micropropagating *Silphium Integrifolium* Michx.

Yuqi Chen (✉ chen3729@umn.edu)

University of Minnesota Twin Cities <https://orcid.org/0000-0002-1914-7438>

Jerry Cohen

University of Minnesota Twin Cities

Kevin Smith

University of Minnesota Twin Cities

Renata Pincelli-Souza

University of Minnesota Twin Cities

Research Article

Keywords: Tissue culture, explant, in vitro culture, contamination, sterilization procedure, MS medium

Posted Date: November 1st, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-968198/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Perennial crops are constantly exposed to fungi and bacteria in their environment, and thus explants from field-grown plants are difficult to disinfect for micropropagation because of both endophytic and epiphytic microbes. Field grown *S. integrifolium* plants were potted and grown in the greenhouse for five weeks; new stems and anthers along with seeds were tested for *in vitro* sterilization. We report successful sterilization protocols that involved disinfecting seeds with isothiazolone biocides (PPM) and NaClO and sterilizing stems and anthers through vacuum infiltration of PPM. The establishing of the protocols is useful for subsequent micropropagation and would in this way facilitate *Silphium* breeding and domestication processes.

Introduction

Found in 21 U.S. states, *S. integrifolium integrifolium* Michx. (rosinweed) is a native herbaceous perennial plant closely related to sunflower (Van Tassel et al., 2017). Because of its long-lived nature, high quality forage biomass yield comparable to corn, along with edible seeds with high quality oil akin to domesticated oilseed sunflower, this species has piqued interest in the plant science community for its potential to become a domesticated perennial oilseed/forage crop (Vilela et al., 2018). In addition, *S. integrifolium* possesses a complex and deep root system that allows it to improve soil structure and health as well as provide food to a wide range of native pollinating insects (Vilela et al., 2018). Domestication efforts have been ongoing for longer than 15 years with experimental trials occurring primarily in the Midwest. Currently, *S. integrifolium* is propagated via seed, cuttings, and divisions. As an obligate outcrossing species, growing from seed cannot achieve true-to-type propagation; propagation through cuttings and divisions however is slow, and cannot ensure disease-free production. Previously, there did not exist a sterilization and micropropagation protocol suitable to achieve rapid true-to-type multiplication of *S. integrifolium*. Obtaining significant numbers of elite genotypes obtained from breeding and selection efforts thus has been difficult to obtain since clonal propagation for *S. integrifolium* has been limited to cuttings and division.

Micropropagation is a tissue culture-based technique for the *in vitro* regeneration of whole plants from organs, tissues, cells or protoplasts (Beverdors, 1990) Micropropagation is dependent on the *in vitro* aseptic culture of cells, tissues, organs or whole plant under controlled nutritional and environmental conditions in order to produce true-to-type clones of pathogen-free plants (George, 2008; Loyola-Vargas & Ochoa-Alejo, 2018). Perhaps the most important step for culture establishment is the sterilization of the explants. An effective way of preventing bacterial contamination *in vitro* requires the elimination of contaminating microorganisms from the initial plant explants that are introduced into the culture (Mihaljević et al., 2013). The methods for reducing contaminations include the use of explants from donor plants maintained under a strictly sanitary regime, reduction of the size of the initial explants to just the apical meristem and eliminating microbial contaminants by pretreatment with one or more chemical antiseptics as well as using antibiotics when explants are cultured on a nutrient medium (George, 2008; Mihaljević et al., 2013).

Perennial crops can have endophytes in specific tissues or can be infected systemically. Endophytes are mainly bacteria entering through natural openings on the plant surface, or through wounds (Cassells, 2012) although fungal endophytes are also known (Waqas et al., 2015). Surface sterilization is not always sufficient for field-grown plants if endophytic contamination is detected, and antibiotics and/or fungicides may be added to the culture medium as an attempt to eliminate the contaminant (Cassells, 2012; Shields et al., 1984). Many factors can determine the success of sterilization, including the types of sterilizing agent(s) used and their concentrations, the period of exposure of the plant tissue to the sterilization agent(s), and the plant organ type used (crown buds, stems, leaves, etc.). Some sterilizing agents include ethanol, sodium hypochlorite (bleach), hydrogen peroxide, and Plant Preservative Mixture PPM, a proprietary mixture of two broad-spectrum isothiazolone biocides. Although it is difficult to eradicate endophytic bacteria with PPM because of the time required for uptake, greater absorption of PPM can be achieved through vacuum infiltration, which allows the sterilizing compound to penetrate deeper into tissues than occurs by simply soaking tissues in the sterilizing compound (Tague et al., 2006). PPM was also found to infiltrate younger axillary buds more effectively, potentially because younger plant tissues are both thinner and less dense (Miyazaki et al., 2010).

Because of the convenience and the potential of decreasing the breeding cycle, *in vitro* techniques are now applied extensively in many breeding programs; however, the lack of well-established *in vitro* techniques often limits more extensive practical uses especially with newer species (Zulkarnain et al., 2015). At present, another species in the *Silphium* genus, *Silphium perfoliatum* L. (cup plant), has been studied regarding the processes required for seed sterilization and micropropagation from apical explants of seedlings in *in vitro* cultures (Tomaszewska-Sowa & Figas, 2011). But, in contrast, there does not exist a *S. integrifolium* micropropagation protocol. This current research aims to establish a *S. integrifolium* tissue sterilization protocol for stems (for clonal propagation), seeds (for embryo rescue or callus culture), and anthers (for anther culture), which would be useful for future research on *S. integrifolium* micropropagation and related tissue culture methods.

Materials And Methods

S. integrifolium's stems, seed, and anthers were utilized in this work. In July 2021, three-year-old field-grown plants were dug out of the soil from field plots located in the St. Paul campus of the University of Minnesota, MN and immediately, washed, the top 25 percent of their stems were trimmed off, and they were planted in 12-in. pots with SunGrow professional sterile potting mix and set to grow in the greenhouse at 90F. After five weeks, the new stems that were produced were cut into 4 cm long pieces with two nodes and utilized for sterilization protocols. Seed and anther tissues were also collected from other greenhouse-grown plants grown in the same conditions. MS medium (Murashige and Skoog, 1962), with 15 g of sucrose and 2 ml of PPM per liter was used as the growth media. Solutions for sterilization contained plant preservative media (PPM) which is composed of 5-chloro-2-methyl-3(2H)-isothiazolone and 2-methyl-3(2H)-isothiazolone (Plant Cell Technology, Washington, DC) ethanol, Softsoap antibacterial soap (Colgate-Palmolive, New York City, NY), commercial bleach (6% NaClO, Clorox, Oakland,

CA) 20%, hydrogen peroxide (H₂O₂), and 10%, Polysorbate 80 (Tween 80, a nonionic surfactant wetting agent to decrease surface tension). All plant tissues were grown in petri dishes with sterile MS medium after sterilization for one month in growth chambers at 25°C with a 12-hour light photoperiod at 28.5 μmol.m.²s⁻¹ of light intensity supplied by cool white florescent tubes to promote growth and development.

Procedure for sterilizing stems

1. Removed all leaves from stems except leaf primordia near the meristem of each stem.
2. Washed tissues with antibacterial soap and 10% alcohol for 5 minutes.
3. [Alternative step] Vacuum infiltrated stems with 2ml/L PPM for 3 minutes at 600 Torr. This step can be skipped if choosing to do step 9.
4. Washed stems with 2ml/L of PPM in double-distilled water and gently shaken by hand for 1 minute.
5. Washed with 1% antibacterial soap for 1 minute; rinsed twice with 2 ml/L PPM.
6. Poured 70% ethanol over stems for 5 seconds in sterile sieve; rinsed again with 2 ml/L PPM.
7. Placed stems into 20% bleach with 1 added drop of Tween 80, and gently shook periodically for 20 minutes.
8. Rinsed with 2 ml/L PPM.
9. [Alternative step] Placed into 10% H₂O₂ for 10 minutes followed with a final wash with 2 ml/L PPM. This step can be skipped if step 3 (vacuum infiltration) was performed.
10. Placed onto sterile MS culture medium.

Procedure for sterilizing seeds

This procedure was essentially the same as the above procedure for sterilizing stems but without the vacuum infiltration nor H₂O₂ treatments. The seed coat was removed before sterilization.

Procedure for sterilizing anthers

1. Using sterile forceps, removed anthers from an unopened or slightly opened *S. integrifolium* flower bud.
2. Washed with 2 ml/L PPM for 1 minute, and then washed with 1% antibacterial soap for 1 minute.
3. Placed into 20% bleach solution with 1 drop (~20 μl) of Tween 80 added and gently swirled periodically for 20 minutes.
4. Washed with 2 ml/L PPM for 10 seconds, dipped into 70% ethanol for 5 seconds, and followed with a final wash with 2ml/L PPM for 10 seconds.
5. Placed onto sterile MS culture medium.

Results

After being grown in the growth chamber for 5 weeks, all stems sterilized with this protocol were still clean and showed no visible signs of microbial contamination. Many had begun growing primordial leaves with the stems displaying phototropic bending and rooted in the media, indicating that the

sterilization procedure was effective (see Fig. 1). However, a small amount of necrosis was present on some stems which possibly occurred as a result of the 10% H₂O₂ treatment. To test this possibility, the stem sterilization experiment was repeated using the stem materials from the same plants, and the H₂O₂ was replaced with vacuum infiltration of 2% PPM in the beginning of the protocol; stem tissues resulting from vacuum infiltration exhibited a 100% success (no colonies of bacteria/fungi visible) without tissue damage. Seeds and anthers sterilized with the described protocols remained without observable contamination, with seeds germinating on their plates after stratification for 4 weeks (see Fig. 2) and anthers remaining viable (not desiccated nor necrotic) for transfer onto growth media containing hormones such as auxins and cytokinins.

Discussion

The protocols to obtain microbe-free stem, seed, and anther tissue in *S. integrifolium* will facilitate future research on micropropagation and *in vitro* mutagenesis using these tissues. They are not dissimilar from the micropropagation protocol proposed for a related species, *S. perfoliatum*, which achieved good results with 30% bleach and 70% ethanol as the main agents in the seed sterilization process (Tomaszewska-Sowa & Figas, 2011); however, that procedure was limited to seeds which are of limited use in the development of elite germplasm lines, whereas this current protocol was shown to be effective with stems and anther tissues as explants, which would thus allow for clonal propagation as opposed to the seed-based protocol used in the *S. perfoliatum* experiments. Any sterilization protocol potentially may need to be adapted for plants grown in other environments or for genotypes with quite different morphologies. Such difference can influence physical behavior during sterilization or, for plants grown under quite different conditions; it may affect external or endophytic microbial populations. Plant tissues used in these experiments were obtained from plants grown initially in fields in the upper Midwest in North America and are thus from a more or less uniform source. Although genetic and extrinsic difference can vary (i.e. characteristics such as stem size, presence, and size of trichomes on the stem), these protocols provide useful procedures that can be optimized for individual differences should changes prove necessary.

The stem sterilization protocol we utilized included taking plants from the field and greenhouse acclimating the plants that produced shoots in the greenhouse over a five-week period. However, the field-grown plants in this experiment produce new shoots within a week in the greenhouse. Thus, it is possible that the shoots produced within the period one week of transplant or longer may be suitable should a more rapid field to culture time be desired. Similarly, although the sterilized seeds required stratification for germination after being sterilized, the dormancy requirement may be overcome by incorporating a growth regulator such as Ethephon (an ethylene-release agent) into the sterile growing media (Reinert et al., 2018). By combining methods such as reducing seed dormancy periods as well as tissue culture micropropagation utilizing this protocol, the breeding process of *Silphium* may be shortened considerably.

Declaration

Data availability statement

The data generated in this protocol are available from Yuqi Chen on reasonable request (chen3729@umn.edu).

References

1. Beversdorf WD (1990) Micropropagation in crop species. In: Nijkamp HJJ, Van Der Plas LHW, Van Aartrijk J (eds) *Progress in Plant Cellular and Molecular Biology*. Kluwer Academic Publishers, Dordrecht, pp 3–12
2. Cassells AC (2012) Pathogen and biological contamination. In: Loyola-Vargas VM, Vazquez-Flota F (eds) *Plant cell culture protocols*, 3rd edn. Humana Press, New York, pp 57–80
3. George EF (2008) Plant tissue culture procedure-Background. In: George EF, Hall MA, De Klerk GJ (eds) *Plant propagation by tissue culture 3rd ed*. Springer, Dordrecht, pp 1–28
4. Loyola-Vargas VM, Ochoa-Alejo N (2018) An Introduction to Plant Tissue Culture: Advances and Perspectives. *Methods Mol Biol* 1815:3–13. doi:10.1007/978-1-4939-8594-4_1
5. Mihaljević I, Dugalić K, Tomaš V, Viljevac M, Pranjić A, Čmelik Z, ... Jurković Z (2013) *In vitro* sterilization procedures for micropropagation of 'Oblačinska'sour cherry. *Journal of Agricultural Sciences, Belgrade*, 58(2), 117-126
6. Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassay with tobacco tissue cultures. *Physiol Plant* 15:473–497
7. Miyazaki J, Tan BH, Errington S (2010) Eradication of endophytic bacteria via treatment for axillary buds of *Petunia hybrida* using Plant Preservative Mixture (PPM™). *Plant Cell Tiss Organ Cult* 102(3):365–372. DOI 10.1007/s11240-010-9741-5
8. Reinert S, Money KL, Rockstad GB, Kane NC, Van Tassel DL, Hulke BS (2018) Two contrasting laboratory methods improve *Silphium integrifolium* Michx. germination rate to agronomically acceptable levels. *Euphytica* 214(9):1–9
9. Shields R, Robinson SJ, Anslow PA (1984) Use of fungicides in plant tissue culture. *Plant Cell Rep* 3(1):33–36. DOI:10.1007/BF00270226
10. Tague BW, Mantis J (2006) In planta *Agrobacterium*-mediated transformation by vacuum infiltration. In: *Arabidopsis protocols*. Humana Press, pp 215–223
11. Tomaszewska-Sowa M, Figas A (2011) Optimization of the processes of sterilization and micropropagation of cup plant (*Silphium perfoliatum* L.) from apical explants of seedlings in in vitro cultures. *Acta Agrobotanica*, 64(4)
12. Van Tassel D, Albrecht KA, Bever JD, Boe AA, Brandvain Y, Crews TE, ... Speranza PR (2017) Accelerating *S. integrifolium* domestication: An opportunity to develop new crop ideotypes and breeding strategies informed by multiple disciplines

13. Vilela A, González-Paleo L, Turner K, Peterson K, Ravetta D, Crews TE, Van Tassel D (2018) Progress and bottlenecks in the early domestication of the perennial oilseed *S. integrifolium* integrifolium, a sunflower substitute. *Sustainability* 10(3):638
14. Waqas M, Khan AL, Hamayun M, Shahzad R, Kang S-M, Kim J-G, Lee I-J (2015) Endophytic fungi promote plant growth and mitigate the adverse effects of stem rot: an example of *Penicillium citrinum* and *Aspergillus terreus*. *J Plant Interact* 10(1):280–287.
DOI:10.1080/17429145.2015.1079743
15. Zulkarnain Z, Tapingkae T, Taji A (2015) Applications of *in vitro* techniques in plant breeding. In J.M. Al-Khayri et al. (eds.), *Advances in Plant Breeding Strategies: Breeding, Biotechnology and Molecular Tools* (pp.293-328). DOI 10.1007/978-3-319-22521-0_10

Figures



Figure 1

Sterilized stems growing in vitro

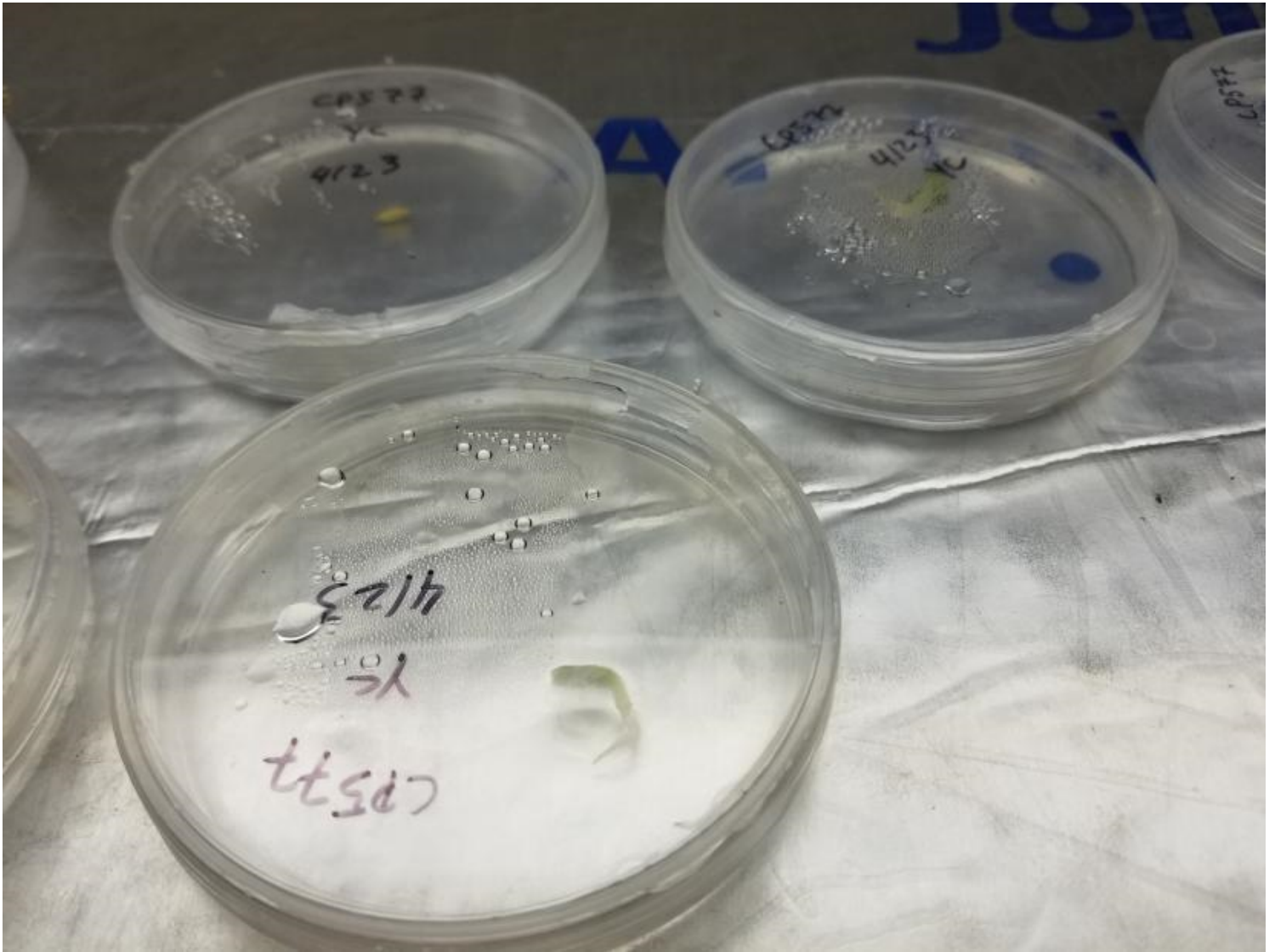


Figure 2

Sterilized seeds germinating in vitro