Improving culture initiation of mature oak shoots through use of silver thiosulfate

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Abstract
Premise: Of the approximately 430 species of oaks (Quercus spp.) that have been assessed, 31% are threatened with extinction and in need of safeguarding. However, oak seeds cannot be seed banked, and thus rely on alternative strategies such as in vitro culture for ex situ conservation. One challenge to this approach is low culture initiation rates. Our objective was to identify factors that may improve the establishment of shoot cultures in vitro using new growth collected from mature trees.

Methods: Shoot cuttings were harvested from individuals of five different oak species (Q. alba, Q. bicolor, Q. macrocarpa, Q. muehlenbergii, and Q. palustris). Shoots were cultured onto medium with or without 50 µM silver thiosulfate (STS), a known inhibitor of the stress hormone ethylene. Cultures were grown for one month, at which point shoots were assessed for survival.

Results: Shoot survival was significantly greater in shoots cultured on medium containing STS compared to the control group, with the overall survival rate increasing from 65% to 73%.

Discussion: Increasing the survival rate of newly established cultures is important in ensuring that material collected from endangered species has the best chance for survival, which is critical for successful ex situ conservation.

KEYWORDS
ex situ conservation, in vitro culture, Quercus, silver thiosulfate

Oaks (Quercus L. spp.) are iconic woody species that are vital members of the many ecosystems they inhabit. However, a variety of threats such as habitat loss and climate change have caused a number of species to decline around the globe (Carrero et al., 2020). Of the 430 species of oaks assessed in the most recent report by the International Union for Conservation of Nature (IUCN), 31% are estimated to be threatened with extinction (Carrero et al., 2020). While in situ conservation methods such as habitat protection are crucial to prevent loss of these species, ex situ strategies are equally important in ensuring their long-term survival by acting as a fail-safe in the event that in situ conservation is unsuccessful (Philpott et al., 2022). The most common ex situ strategy for many angiosperms is seed banking, where seeds collected from wild populations are dried and kept at −20°C, allowing them to be stored potentially for decades (Walters and Pence, 2021). However, oak seeds (acorns) are intolerant of the desiccation necessary for seed banking, and thus are classified as “exceptional species” (i.e., they cannot be conserved ex situ using conventional seed-banking methods; Kramer and Pence, 2012; Pence and Chaiken, 2021; Pence et al., 2022). Therefore, alternative strategies that target different germplasm such as in vitro tissue culture and cryopreservation represent the most viable method of ex situ conservation for this genus (Walters et al., 2013; Pence et al., 2020). For long-term storage of oak germplasm, two methods that have previously been demonstrated to be effective are cryopreservation of somatic embryos and cryopreservation of shoot tips (Sánchez et al., 2008; Pence and Chaiken, 2021). However, neither of these methods are available until an in vitro culture line has been established to serve as a tissue
source, making this step a crucial part of the conservation process.

In vitro culturing is not without its own challenges, however (Pence, 2013). Material harvested from wild individuals does not always respond well to being cultured, and survival of oak shoots in vitro can often be quite low (Johnson and Walker, 1990; Preece and Compton, 1991). Even if shoot survival is initially high, it may decrease significantly over time (Kramer and Pence, 2012; Brennan et al., 2017). The stress of being cut from the mother plant and exposure to sterilizing agents such as sodium hypochlorite combined with the frequent presence of endophytes (Volk et al., 2022) can often lead to necrosis of the tissue and the exudation of phenolic compounds that oaks are known to produce (Garcia-Gonzáles et al., 2010; Custódio et al., 2015; Martínez et al., 2021). This is particularly damaging when working with rare or threatened species, as wild populations may only have a limited number of shoots that can be safely collected in a given year. In other cases, species that produce little to no seed may have few, if any, juvenile individuals present in the population, and so material may need to be collected from mature individuals, which are more prone to phenolic exudation and oxidation and typically have lower survival rates in culture (Shekhawat et al., 1993; García-Gonzáles et al., 2010). The recalcitrance of mature material is a significant challenge to the in vitro propagation of many woody species (McCown, 2000; Bonga et al., 2010). Determining how to address these challenges in order to maximize the survival of collected shoots is therefore paramount to ensuring that ex situ conservation efforts are successful.

The purpose of this study was to determine whether modifications could be made to the culture medium to improve the survival rate of freshly cultured oak shoots collected from mature trees. It is known that ethylene can be produced by tissues under stress and can, in turn, negatively impact tissue growth, usually through necrosis and leaf abscission (Beyer, 1979; Veen, 1983; Reid, 1985; Gaspar et al., 1996; Kumar et al., 1998; Pech et al., 2002; Park et al., 2016). To circumvent the negative effects that ethylene can have on cultured tissue, one option is to add the silver ion (Ag⁺), a known inhibitor of ethylene action, either in a thiosulfate form (STS) or as AgNO₃ (Beyer, 1976; Veen, 1983; Gaspar et al., 1996). STS has previously been used to influence the in vitro growth and development of several woody species (Steinitz et al., 2010; San et al., 2015; Diab, 2017). It has also been tested on oaks and found to reduce shoot tip necrosis and leaf senescence in newly initiated cultures of the European species *Q. ilex* L. (Martínez et al., 2017). In North American oak species, STS has been used to improve somatic embryogenesis (Martínez et al., 2015). Although there have been other instances of ethylene inhibitors such as AgNO₃ being used to improve oak culture multiplication, again by reducing shoot tip necrosis (Vieitez et al., 2009), it has been suggested that STS may be a more effective source of Ag⁺ in the culturing medium (Gaspar et al., 1996). Our objective was to test whether the inclusion of STS in the culture medium could improve the in vitro survival of shoots collected from mature individuals of multiple *Quercus* species. It was hypothesized that inhibiting the action of ethylene would help prevent senescence of cultured tissue and promote shoot survival and regeneration.

**METHODS**

**Material collection**

Branches with new growth were collected from mature individuals over the course of three years (2018–2020) during sampling periods in late April and early May. Five species were represented (*Q. alba* L., *Q. bicolor* Willd., *Q. macrocarpa* Michx., *Q. muehlenbergii* Engelm., and *Q. palustris* Münchh.), which were collected from the Cincinnati Zoo & Botanical Garden, Spring Grove Cemetery and Arboretum, and The Morton Arboretum (see Appendix 1). One individual (i.e., one genotype) was sampled from *Q. alba*, *Q. macrocarpa*, and *Q. palustris*. Two individuals of *Q. muehlenbergii* were sampled, one in 2019 and another in 2020, and three individuals of *Q. bicolor* were sampled, one in 2019 and two in 2020. Previous testing done by our lab found that shoots that had grown at least 1 cm in length but that lacked fully expanded and/or hardened leaves were the most likely to respond to culturing, so for this study branches were selected to obtain shoots at this stage (Figure 1). Once cut, branches from each individual were placed into buckets of water and kept in cold storage (4°C) for up to seven days until shoots could be harvested and cultured. The number of shoots collected per individual ranged from 40 to 200, depending on the stage of shoots at the time of collection and the availability of accessible branches (see Table 1).

**Culture initiation**

Harvested shoots were defoliated and then sterilized by swirling all shoots from a given species together in a 1:10 dilution of commercial bleach with 0.05% Tween 20 (Product J20605.AP, Thermo Scientific, Fair Lawn, New Jersey, USA) for 10 min. After sterilization, the shoots were rinsed in sterilized (autoclaved) reverse osmosis water. Shoot ends were trimmed, both to remove bleached ends and to roughly homogenize shoot length (to ~1 cm). Shoots were then placed into individual 50-mL test tubes (Figure 2) with approximately 15 mL of Woody Plant Medium (Lloyd and McCown, 1980) with Murashige and Skoog (1962) vitamins (Product M553, PhytoTech Labs, Lenexa, Kansas, USA), 0.2 mg/L benzylaminopurine, 3% sucrose, and 0.25% gellan gum (Gelzan, PhytoTech Labs Product G3251). Half of the shoots were placed onto the aforementioned medium (as a control) and the other half were placed onto the same.
medium with the addition of 50 µM STS, which was filter sterilized and added to the medium after autoclaving. To ensure maximum efficacy, both the STS solution and the medium containing it were made within 24–48 h of the shoots being cultured.

Growing conditions

Shoot cultures were grown in an incubation room under low light (10–20 µmol m⁻² s⁻¹ photosynthetically active radiation [PAR]) with a 16:8 h light:dark cycle and at approximately 25°C. During the incubation period, shoots were routinely checked for contamination, and those that were found to be significantly contaminated (completely covered by either bacterial or fungal growth) were discarded and not included in the final analysis.

Data analysis

After one month, shoots were evaluated for survival (defined as the presence of a bud or node that was still green) using a dissecting scope. Because the majority of culture failure occurs within the first few weeks after initiation (Brennan et al., 2017), the one-month mark was chosen as the data collection point to obtain a more accurate representation of shoot response. Overall survival of shoots on both the control and the STS media was first compared across all species with a two-proportion Z-test, using R version 4.1.0 (R Core Team, 2021) in RStudio version 1.4.1717 (RStudio Team, 2021). The contamination rate of cultures was compared using a sign test at α = 0.05. Survival of shoots on the control and STS media was then compared for each individual species, and for all species overall, using a test of homogeneity of odds ratios following the Mantel–Haenszel procedure (Sokal and Rohlf, 1995).

For four species in which samples were tested over two years (Q. alba and Q. palustris) or that included multiple genotypes (Q. bicolor and Q. muehlenbergii), survival data were separated by year and/or genotype for analysis. One species (Q. macrocarpa) was only sampled in a single year. For the combined species analysis, each species was represented by data combined over multiple years or genotypes.

RESULTS

Overall, the average survival of shoots significantly increased when shoots were placed onto medium containing STS (73%) compared to the control medium (65%; two-proportion Z-test; $\chi^2 = 7.458, df = 1, P = 0.0063$; Figure 3). The contamination rate of cultures varied widely in both directions, and overall was found not to be significantly different. When compared across all five Quercus species, the odds of a shoot surviving in culture was 1.1 times greater for shoots cultured on medium containing STS compared to the control medium (odds ratio = 7.02, df = 4, $P = 0.0099$). However, the responses of individual species were mixed, both in terms of the difference (or lack thereof) in survival rate between the control and STS groups as well as the baseline survival rate overall (Figure 4). Survival rates of shoots on the control medium ranged from as low as 10% up to 96%, while survival rates of shoots on medium containing STS ranged from 27% up to 99% (Table 1). Shoots of Q. alba exhibited a significant increase in survival when placed onto STS medium in both years (mean = 61.7%; odds ratio = 8.89, df = 1, $P = 0.0117$), with survival being 2.94 greater than the control treatment alone (32.5%). Quercus bicolor also showed an increase in survival on STS medium (81.5%) over 2.0 times compared to the control treatment (67.7%), but this difference was not statistically significant (odds ratio = 5.09; df = 2, $P = 0.0784$). Quercus palustris shoots cultured onto media containing STS had a small but nonsignificant increase in survival (96.2% vs. 89.6%; odds ratio = 2.26, df = 1, $P = 0.3234$). In contrast, shoots of Q. muehlenbergii exhibited conflicting trends, with survival increasing on medium with STS during the first year but decreasing the second year ($P > 0.05$; Table 1). Quercus macrocarpa, the only species tested in a single year, exhibited a slight but nonsignificant decrease in survival.
TABLE 1  Comparison of the individual survival and contamination rates of sampled *Quercus* species cultured on media with or without silver thiosulfate (STS), along with the sample size of each group (shoots that were significantly contaminated were discarded and not included in the final counts). The table is organized by year, and then by species. Species for which multiple individuals were collected are marked with an identifying number.

| Year | Species/Genotype | STS | Sample size | Survival | Contamination |
|------|------------------|-----|-------------|----------|---------------|
|      |                  |     |             |          |               |
| 2018 | *Q. alba* (−)    | 20  | 10.0%       | 5.0%     |
|      |                  | 20  | 50.0%       | 10.0%    |
|      | *Q. bicolor* (1) | 30  | 56.7%       | 30.0%    |
|      |                  | 30  | 70.0%       | 36.7%    |
|      | *Q. palustris*   | 30  | 83.3%       | 10.0%    |
|      |                  | 30  | 93.3%       | 13.3%    |
| 2019 | *Q. alba*        | 60  | 55.0%       | 78.3%    |
|      |                  | 60  | 73.3%       | 71.7%    |
|      | *Q. palustris*   | 98  | 95.9%       | 72.5%    |
|      |                  | 99  | 99.0%       | 43.4%    |
|      | *Q. muehlenbergii* (1) | 99 | 43.4% | 24.2% |
|      |                  | 99 | 47.5%       | 9.1%     |
| 2020 | *Q. bicolor* (2) | 38  | 73.7%       | 5.3%     |
|      |                  | 39  | 94.9%       | 5.1%     |
|      | *Q. bicolor* (3) | 70  | 72.9%       | 14.3%    |
|      |                  | 69  | 79.7%       | 39.1%    |
|      | *Q. muehlenbergii* (2) | 33 | 36.4% | 33.3% |
|      |                  | 33 | 27.3%       | 9.1%     |
|      | *Q. macrocarpa*  | 36  | 83.3%       | 22.2%    |
|      |                  | 33  | 78.8%       | 48.5%    |

FIGURE 2  After sterilization, shoots were trimmed to homogenize shoot length and then placed into individual test tubes containing either the control or the STS medium.
Improving the survival of collected shoots is absolutely crucial to ensuring successful ex situ conservation as either in vitro cultures or cryopreserved samples. The results of this study show promise in achieving that goal, at least for the majority of Quercus species examined here. However, all of the species used in this study, except for Q. palustris, were white oaks (Quercus sect. Quercus). While Q. palustris did show an increase in survival when placed onto medium containing STS, the differences were not statistically significant. On the other hand, Q. palustris had the highest survival rate of the five species tested, averaging above 90%. Consequently, the presence of an ethylene inhibitor like STS may not be very beneficial when the baseline survival rate is already so high. Given the variation in responses between the various white oak species, it would be beneficial to investigate whether the response exhibited by Q. palustris is the same for other red oaks. Therefore, future work should expand to not only include more species, but specifically include additional species from the red oaks (Quercus sect. Lobatae). Additionally, it would be useful to investigate whether the concentration of STS in the medium has any significant effect on shoot survival. The concentration used in this study was 50 µM, which was determined to be the optimum concentration for another species studied in this laboratory and is more than twice the concentration (20 µM) used in the previously referenced study (Martínez et al., 2015). However, establishing a gradient to determine the optimum concentration of STS
could prove exceptionally useful, as the concentration we used here may not have been sufficient depending on the amount of ethylene the shoots produced.

Future investigations should also consider additional variables suggested in the current study that could potentially affect shoot survival in *Quercus*. For example, the size and age of the source individual may be important. In the current study, the sampled individual that exhibited the greatest difference in survival rates (*Q. alba*) with treatment was also markedly the oldest at an estimated 400 years old. Given the recalcitrance of mature material (McCown, 2000), the efficacy and benefit of ethylene inhibitors like STS may increase significantly with the age of the source tree. In addition, the survival responses exhibited within *Quercus* species in this study may indicate temporal variability from year to year and/or genotypic variability reflecting the different genotypes present. For example, while all three of the *Q. bicolor* genotypes tested exhibited very similar responses (including across different years), the two *Q. muehlenbergii* genotypes responded in opposite ways. Although it is difficult to draw definite conclusions given that only two of the five examined species included more than one genotype, and those were sampled in different years, the effect of genotype could have a significant influence on the response of cultured tissues and should be examined further.

Previous research on the initiation of oak cultures has primarily focused on testing different media formulations and manipulating hormone concentrations (Johnson and Walker, 1990; Chalupa, 1993; Puddephat et al., 1997; Fadladeen and Toma, 2020). However, the inclusion of additional compounds such as antioxidants and hormone inhibitors could potentially offer new insights and address certain problems that prevent successful culture initiation. As these compounds continue to be tested and their particular benefits identified, we will continue to progress toward in vitro tissue culture becoming a secure and reliable strategy for ex situ conservation of *Quercus* species, especially involving mature trees. Overall, the use of STS in the culturing medium appears to demonstrate applicable benefits that may improve our ability to effectively conserve these tree species, especially when seed propagation is not an option.

**AUTHOR CONTRIBUTIONS**

M.W. and V.C.P. planned and designed the research. M.W. performed the experiment and collected all of the data. T.C. performed the data analysis. M.W. created the figures and
IMPROVING CULTURE INITIATION OF MATURE OAK SHOOTS

wrote the first draft of the manuscript, which V.C.P. and T.C. edited before submission. M.W. made all of the final edits prior to publication. All authors approved the final version of the manuscript.

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OPEN DATA BADGE

This article has earned an Open Data Badge for making publicly available the digitally shareable data necessary to reproduce the reported results. The data are available at https://doi.org/10.17632/rrb9289jbw.1. Learn more about the Open Practices badges from the Center for Open Science: https://osf.io/tvyxz/wiki.

DATA AVAILABILITY STATEMENT
Data files and analyses have been uploaded to the Open Science Framework and are accessible at https://osf.io/j3euw/ (Winkeljohn et al., 2022).

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**APPENDIX 1** Collection records for all source material. On-site location has been provided for trees that do not have an individual accession number.

| Year | Genus | Species | Voucher no. (Herbarium)* | Location | Accession no./[Location] |
|------|-------|---------|--------------------------|----------|--------------------------|
| 2018 | Quercus | alba | MW0002 (CINC) | Spring Grove Cemetery & Arboretum | [Section 101] |
| 2018 | Quercus | bicolor | MW0001 (CINC) | Spring Grove Cemetery & Arboretum | [Section 21] |
| 2018 | Quercus | palustris | MW0003 (CINC) | Spring Grove Cemetery & Arboretum | [Section 132] |
| 2019 | Quercus | alba | MW0002 (CINC) | Spring Grove Cemetery & Arboretum | [Section 101] |
| 2019 | Quercus | palustris | MW0003 (CINC) | Spring Grove Cemetery & Arboretum | [Section 132] |
| 2019 | Quercus | muehlenbergii | — | Morton Arboretum | 704–46*3 |
| 2020 | Quercus | bicolor | MW0004 (CINC) | Cincinnati Zoo & Botanical Garden | 060044-01 |
| 2020 | Quercus | muehlenbergii | MW0007 (CINC) | Cincinnati Zoo & Botanical Garden | 060155-03 |
| 2020 | Quercus | macrocarpa | MW0006 (CINC) | Cincinnati Zoo & Botanical Garden | 050002-06 |
| 2020 | Quercus | bicolor | MW0005 (CINC) | Cincinnati Zoo & Botanical Garden | 060044-03 |

*Herbarium acronyms are per Index Herbariorum (Thiers, 2022).*