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METHOD PAPER

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# Simple methods to remove microbes from leaf surfaces

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## Abstract

Endophytes have been defined as microorganisms living inside plant tissues without causing negative effects on their hosts. Endophytic microbes have been extensively studied for their plant growth-promoting traits. However, analyses of endophytes require complete removal of epiphytic microorganisms. We found that the established tests to evaluate surface sterility, polymerase chain reaction, and leaf imprints, are unreliable. Therefore, we used scanning electron microscopy (SEM) as an additional assessment of epiphyte removal. We used a diverse suite of sterilization protocols to remove epiphytic microorganisms from the leaves of a gymnosperm and an angiosperm tree to test the influence of leaf morphology on the efficacy of these methods. Additionally, leaf tissue damage was also evaluated by SEM, as damaging the leaves might have an impact on endophytes and could lead to inaccurate assessment of endophytic communities. Our study indicates, that complete removal of the leaf cuticle by the sterilization technique assures loss of epiphytic microbes, and that leaves of different tree species may require different sterilization protocols. Furthermore, our study demonstrates the importance of choosing the appropriate sterilization protocol to prevent erroneous interpretation of host-endophyte interactions. Moreover, it shows the utility of SEM for evaluating the effectiveness of surface sterilization methods and their impact on leaf tissue integrity.

**KEYWORDS**

endophyte, leaf surface sterilization, *Pinus contorta*, *Populus fremontii*, scanning electron microscopy (SEM)

Plant endophytes are described as microorganisms living inside plant tissues without deteriorating their hosts' health [1–3]. In recent years, studies of plant-endophyte interactions have gained considerable attention due to the potential contributions of these interactions to improve plant health [4–9]. However, several methodological obstacles arise when studying endophytes, including the complete removal of leaf surface microorganisms (i.e., leaf epiphytes). Current methods used to confirm leaf surface sterility generally include polymerase chain reaction (PCR) and leaf prints on nutrient media [10–18]. We included scanning electronic microscopy (SEM) to improve the validation of leaf surface sterility and to evaluate the potential damage by these treatments on the integrity of leaf tissue. Using SEM, we found that PCR and leaf imprinting results are not always indicative of leaf sterility. Furthermore, our microscopic analyses indicated that different plant species may require different

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sterilization methods to remove all surface microorganisms without significantly damaging leaf tissue.

Here, we describe simple techniques that we have reviewed to remove leaf epiphytic microorganisms. To demonstrate these approaches, we used leaves from two plant species with different phylogenies and leaf morphologies: the gymnosperm tree *Pinus contorta* Douglas ex Loudon var. *murrayana* (Balf.) Engelm. (Sierra lodgepole pine), and the angiosperm tree *Populus fremontti* S. Watson (Fremont cottonwood).

Cottonwood leaves were collected at the University of California, Merced (37°22′04.5″N, 120°25′21.8″W) and lodgepole pine needles were obtained from Yosemite National Park, CA (37°39′46.9″N, 119°39′38.2″W). The leaves were collected aseptically, placed in sterile bags, and immediately transported to the laboratory at the University of California, Merced, where the leaves were surface sterilized using the procedures described below. The effectiveness of four different sterilization methods was evaluated by PCR and leaf imprinting on a nutrient medium. Additionally, leaf surfaces were analyzed by SEM.

The applications of peroxide, ethanol or bleach alone, or combined with each other, are commonly used to remove leaf epiphytic microorganisms [19–24]. Therefore, the efficacy of these reagents for removing leaf epiphytes was evaluated using 1 g of fresh weight leaf tissue for each of the treatments. The four applied sterilization protocols were followed by two rinses with sterile water for 30 s and were analyzed in triplicate.

The first treatment consisted of a sonication protocol that was successfully applied to remove epiphytic microbes from *Arabidopsis thaliana* roots. In this procedure, sonication shattered the entire root surface and no epiphytic microbes were detected [25]. We tested a similar protocol to evaluate the effect on leaf cuticles by applying a frequency of 40 kHz (Branson M1800 Ultrasonic Cleaner, CT) for 10 min in 1× phosphate-buffered saline (PBS) solution with 0.02% Silwet L-77 (Lehle Seeds, TX). In the second treatment, the leaves were washed for 1 min in 100% ethanol. The third sterilization method consisted of a 5-min wash with 8.25% sodium hypochlorite (i.e., commercial bleach). The fourth treatment was a 1-min wash in 30% hydrogen peroxide.

To evaluate the effectiveness of these four treatments to remove leaf epiphytic bacteria by PCR, the last rinse of treated and control leaves (1 g of fresh weight tissue washed in sterile water for 1 min followed by two 30-s rinses with sterile water) was saved and used for amplification of 16S RNA genes, as described previously [12,20], using primers 27F and 1492R [26,27]. Additionally, treated and nontreated leaves were used to imprint on Lysogeny Broth (LB) media for 30 sec and incubated at 28°C for 3 days [28]. Overall, a minimum of 20 leaves per treatment and plant species was tested by imprinting. Furthermore, SEM was used to visualize the effectiveness of each sterilization protocol. At least 40 images per treatment and plant species were analyzed. Treated and nontreated leaves were immediately transferred to 2.5% glutaraldehyde in 0.1-M PBS at pH 7.0 for fixation [29]. After 24 h, the samples were washed twice in 0.1-M PBS for 15 min. Next, plant tissues were dehydrated through a graded series of 50, 75, 95 (in sterile water), and 100% ethanol solutions for 15 min for each step. Samples were then transferred to a DCP-1 critical point drying apparatus (Denton Vacuum, NJ) using carbon dioxide as the transitional fluid. Afterward, the samples were mounted on aluminum stubs and gold coated with a Polaron SEM Coating Unit E5000 (Bio-Rad, CA). A Zeiss Gemini SEM 500 (Carl Zeiss, Baden-Württemberg, Germany) was used for imaging, operated at an accelerating voltage of 3 kV.

PCR amplification using the final rinses gave negative results for all samples tested, including the control leaves (Figure 1a). Additionally, growth on LB after imprinting was only observed for non-surface-sterilized samples and for the ethanol washed samples from cottonwood (Figure 1b). In contrast, imaging of leaf surfaces using SEM demonstrated that imprinting and PCR are not always reliable methods to evaluate leaf surface sterilization (Figure 2). Removal of epiphytic microbes was based on the loss of hyphae or spherical and ellipsoidal structures with the size of bacteria or yeast cells (1–10 μm). Untreated samples (Figure 2a,b) were compared to the differently treated leaves. Sonicated leaves of both species showed microorganisms remaining on their surfaces (Figure 2c,d), indicating that the protocol successfully used for *Arabidopsis* [25] roots is not suitable for pine and cottonwood leaves. Using ethanol for sterilization was successful in removing surface microbes from lodgepole pine needles (Figure 2e), whereas epiphytes on cottonwood leaves were still present (Figure 2f). To test the feasibility of ethanol as a sterilization reagent for cottonwood leaves, the duration of this treatment was extended to 20 min. Surface microbes could still be visualized by SEM even after these extended washes (Figure S1). For bleach-treated samples, no microbes were observed on the leaves of both species (Figure 2g,h).

Besides the removal of microbes, we also analyzed leaves for tissue damage. Cottonwood leaves were apparently undamaged by bleach treatments (Figure 2h). In contrast, peroxide treatments caused substantial leaf tissue damage, and in most cases, the leaves were in too poor of a condition for mounting to image them. The leaves that survived the treatment were covered with debris that might have originated from damaged

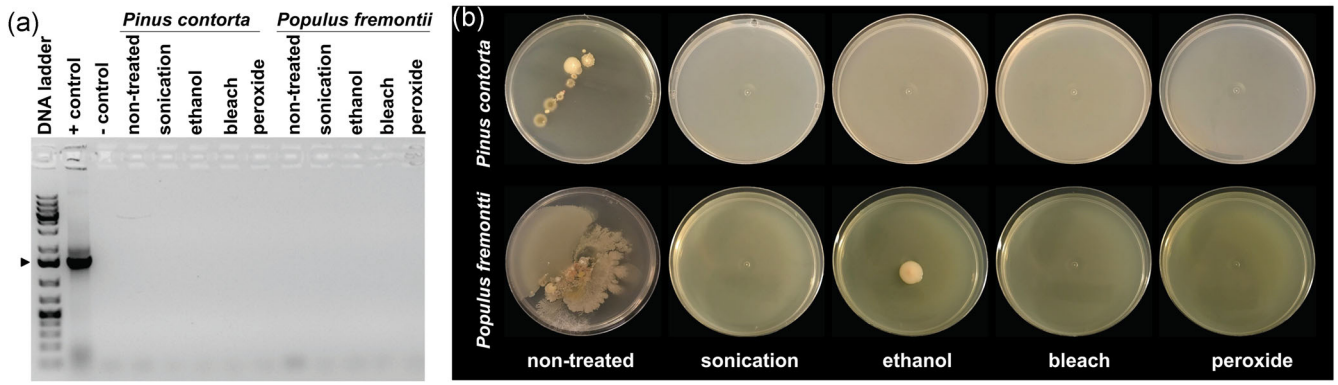


**FIGURE 1** Evaluation of leaf surface sterilization protocols. (a) Amplification of 16S RNA genes. Primers 27F and 1492R were used for amplification. Description *Escherichia coli* DNA and water were used as positive and negative controls, respectively. Black arrowhead indicates the 1.5-kb band of the DNA ladder. (b) Nontreated and treated leaves were imprinted in Lysogeny Broth media

leaf tissue (Figure 2j). Furthermore, pine needles showed ruptures using bleach and peroxide treatments (Figure 2g,i).

Our SEM analyses indicated that removing the cuticle is necessary to achieve leaf surface sterilization, whereas its partial removal led to the formation of cuticle clusters that contained epiphytic microbes (Figure S2). Therefore, we recommend the use of ethanol treatments to achieve the appropriate surface sterilization for lodgepole pine needles and the use of bleach for cottonwood leaves. These respective treatments appeared superior for removing leaf epiphytes because they were effective in removing leaf cuticles without impairing leaf tissue integrity. Additional research is needed to determine, if plant species-specific sterilization treatments are required for effective leaf surface sterilization or if these two treatments for lodgepole pine needles and Fremont cottonwood leaves are generalizable across other species within their respective gymnosperm and angiosperm groups.

Taken together, our study reveals that the PCR and leaf imprints may be insufficient to demonstrate leaf surface sterilization, thus leading to inaccurate conclusions about the structure and function of the leaf endophytic communities. Moreover, we show that different plant species may require different treatments to remove leaf epiphytic microbes without causing significant damage to the leaf tissue; damaging leaf tissue might impact endophytic microbial communities and could lead to erroneous interpretations of microbe–host interactions.

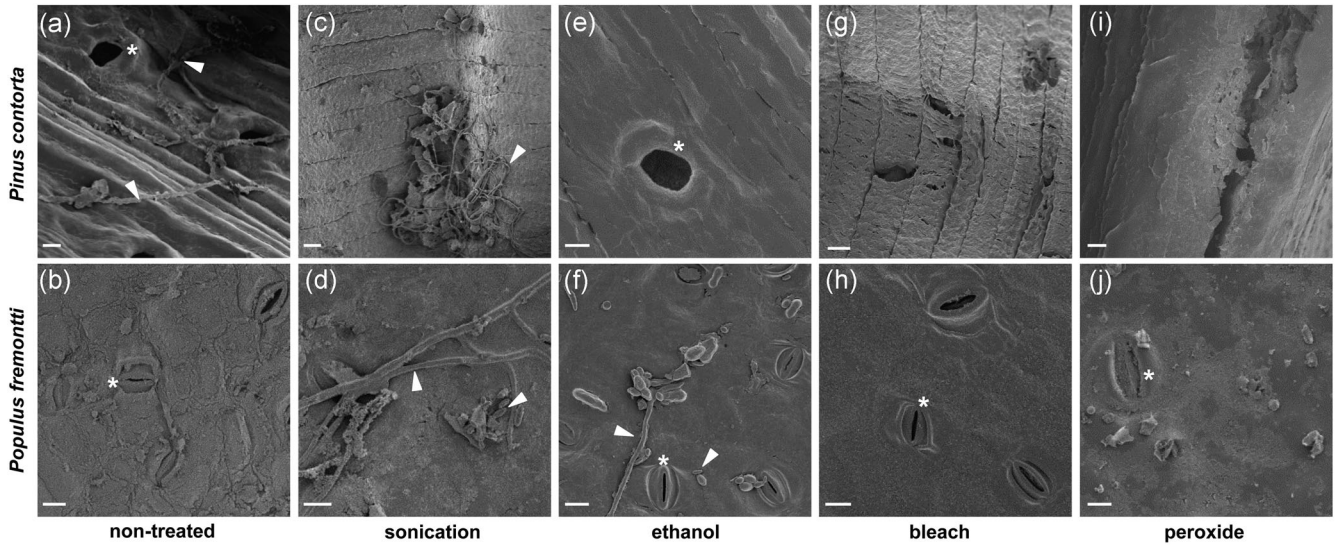


**FIGURE 2** Representative scanning electron microscopy images of leaf surfaces of *Populus fremontti* and *Pinus contorta* before and after applying the different sterilization protocols. White arrowheads point at examples of epiphytic microbes. Asterisks indicate leaf stomata. Scale bar = 10 μm

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## CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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