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Research article

A new method for extracting DNA from the grape berry surface, beginning in the vineyard

Megan E. Hall [a,c,*], Lance Cadle-Davidson [b], Zhiwei Fang [c], Wayne F. Wilcox [a]

[a] Section of Plant Pathology and Plant-Microbe Biology, School of Integrative Plant Science, Cornell University, NYS Agricultural Experiment Station, Geneva, NY, 14456, United States
[b] United States Department of Agriculture–Agricultural Research Service, Grape Genetics Research Unit, Geneva, NY, 14456, United States
[c] Division of Plant Sciences, University of Missouri, Columbia, MO, 65211, United States

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ABSTRACT

Isolating DNA from microbes on the surface of a grape berry is a challenge due to their adhesion to the thick berry skin and cuticle, making studies of the grape microbiome challenging. We developed a field-to-lab DNA extraction procedure that starts in the vineyard, disrupts the grape berry surface while en route to the lab through agitation, and efficiently extracts microbial DNA from the surface of the grape. It is cost effective and utilizes commonly available laboratory chemicals with low toxicity (Table 1). This protocol allows researchers to extract DNA from the grape berry surface in the field, therefore undergoing minimal manipulation of those microbiomes before DNA extraction.

1. Introduction

Metagenomic analysis is now crucial to the study of microbial diversity, but its application in natural environments is problematic due to the need for high quality DNA obtained from less-than-ideal environmental situations. Isolating DNA from the surface of a grape berry involves aggressive and disruptive actions, due to tight adhesion of microbes to the thick berry skin and cuticle, making it difficult to wash microbes off the surface using most commercial kits [1], with some exceptions [2, 3]. More commonly, researchers have used culture-based methods [4, 5, 6, 7, 8, 9] or sampled grape must [10, 11, 12] to conduct microbial ecology studies.

A plethora of DNA extraction methods exists already, ranging from DNA extraction kits to traditional methods, such as CTAB extractions. The efficacy and cost of these techniques vary. Many published methods are not useful for complex plant tissues which often contain inhibitory compounds, or for samples like grape berries that have a challenging surface from which to extract microbes. Furthermore, the process of DNA extraction typically begins in the laboratory after samples have been collected from the field, which increases the likelihood that microbial communities can be altered or disrupted from the time they are collected until the time they are processed. For example, published methods relying upon harsh chemicals, such as phenol or chloroform, require that samples be brought into the lab before the extraction procedure can begin, and additionally raise worker safety concerns. For these reasons, we developed a DNA extraction procedure that starts in the field, efficiently extracts microbial DNA from the surface of the grape, is cost effective, and can be made from commonly available laboratory chemicals with low toxicity.

2. Materials & methods

A grape berry of *Vitis vinifera* cv. Red Globe was excised directly from a cluster into a 50 mL Falcon tube containing 5 mL of a TE buffer (10mM Tris-HCl+1mM EDTA, ph 8.0) solution containing 10% NaCl. 500 μl of 10% SDS was added to the Falcon tube containing the TE-NaCl solution, vortexed for 5 seconds and left at room temperature for 15 min. A freeze-thaw sequence consisting of 30 min in a -80 °C freezer and five min in 60 °C water bath was repeated three times to lyse the fungal and bacterial cells. The solution was then centrifuged at 15000 *g*, 4 C for 10 mins to

* Corresponding author.
E-mail address: hallmegan@missouri.edu (M.E. Hall).

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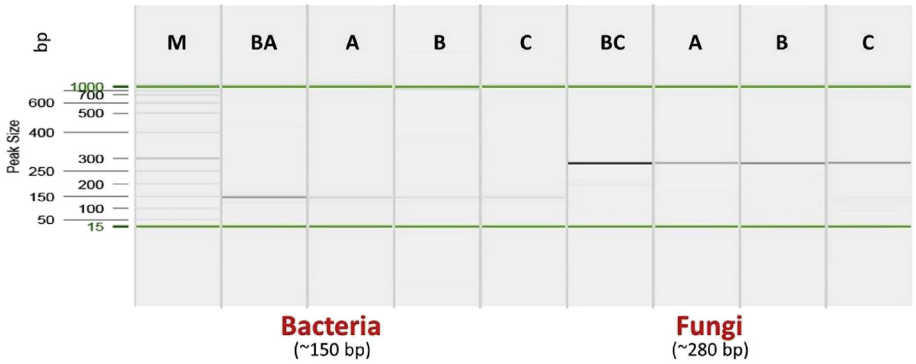

Fig. 1. Comparison of three DNA extraction techniques in the amplification of bacterial and fungal DNA. (M) is the marker; (BA) is the positive bacterial control using *Acetobacter aceti* and extraction method (A); (BC) is the positive fungal control using *Botrytis cinerea* and extraction method (A); (A) is our method of DNA extraction from a grape berry; (B) is the extraction from a grape berry using a standard CTAB extraction; (C) is the extraction from a grape berry using the MoBio PowerSoil commercial kit. All 16S primers generated 150-bp amplicons while the ITS primers generated a 280-bp amplicons.

remove debris and salt-SDS reaction flakes. The supernatant was carefully collected and a 750 µl-aliquot was transferred to a 2 mL centrifuge tube, along with 750 µl ice-cold isopropanol and vortexed for approximately 30 s. The solution was centrifuged for 10 min at 4 C, 17000 *g* (13000 rpm). The supernatant was carefully removed from the tube and the pellet was washed twice with 500 µl of ice-cold 70% ethanol at 17000 *g* (13000 rpm) for 2 m. The pellet was re-suspended in 30 µl TE buffer after it was dried in 50 C heat block for 5–10 m.

For comparison to commonly used protocols, we used a CTAB extraction and the MoBio PowerSoil commercial kit, both with the TE + NaCl and SDS solution. For the CTAB extraction, a 500 µl-aliquot of the supernatant was mixed well with 670 µl of CTAB buffer in a 2 mL centrifuge tube and heated at 65 °C for 30 min. After cooling down to room temperature, it was mixed with 750 µl of phenol:-chloroform:isoamyl alcohol (50:48:2), and vortexed for approximately 20 seconds. It was then centrifuged at room temperature for 5 minutes at 17,000 *g*, then the upper aqueous phase was carefully transferred to a fresh tube and washed with 750 µl of chloroform and centrifuged for 5 m at 17,000 *g*. An equal volume of ice-cold isopropanol was added to the aqueous phase, vortexed for 30 s and centrifuged at 4 C, 17000 *g* (13000 rpm) for 10 min. The DNA pellet was washed twice with 500 µl of ice-cold 70% ethanol at 17000 *g* (13000 rpm) for 2 mins. The pellet was dried in 50 C heat block for 5–10 mins and re-suspended in 20 µl of TE buffer.

For the commercial kit, a 750 µl-aliquot was transferred to a 2 mL centrifuge tube, along with 1200 µl of Solution C4 (Qiagen DNeasy PowerSoil Kit) and mixed well with pipetting. Then it was loaded onto an MB Spin Column and the manual instructions were followed to elute DNA in 30 µl of elution buffer.

As a positive control, we used our own method on a known bacteria (BA), *Acetobacter aceti*, and fungus (BC), *Botrytis cinerea*.

To determine the amount of DNA extracted using each protocol, each protocol was conducted on a single berry in replicates of five. This, in turn, was repeated a total of five times, for a total of 25 DNA extractions per berry. The amount of DNA was calculated using a NanoDrop One Spectrophotometer (Thermo Scientific, Hanover Park, IL).

To amplify the fungal DNA from the berry surface, fungal internal transcribed spacer (ITS) 1 loci were amplified using primers BITS (5′–CTACCTGCGGARGGATCA–3′) and B58S3 (5′–GAGATCCRTTGYTRAAAGTT–3′), and to amplify bacterial DNA, the V4 domain of the bacterial 16S rRNA genes was amplified using primers F515 (5′–GTGTGCCAGCMGCCGCGGTAA–3′) and R806 (5′–GGACTACHVGGGTWTCTAAT–3′) [11]. A polymerase chain reaction (PCR) was performed in 25-µl reaction volumes containing GoTaq® G2 Green Master Mix (Promega Corporation, Madison, WI), 10 mM of each primer and approximately 10 ng genomic DNA. Reactions conditions used to amplify the fungal amplicons consisted of an initial 95 °C for 2 min; followed by 40 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 60 s; and a final extension of 72 °C for 5 min. [11]. All amplifications were performed in a T100 Touch Thermal Cycler (Bio-Rad Laboratories Inc., Hercules, CA). DNA analysis was performed on the QIAxcel Advanced system (Version: 9001421, QIAGEN, Germany) using the OM400 method described in the QIAxcel DNA Handbook. The results were displayed as a gel image using QIAxcel system software.

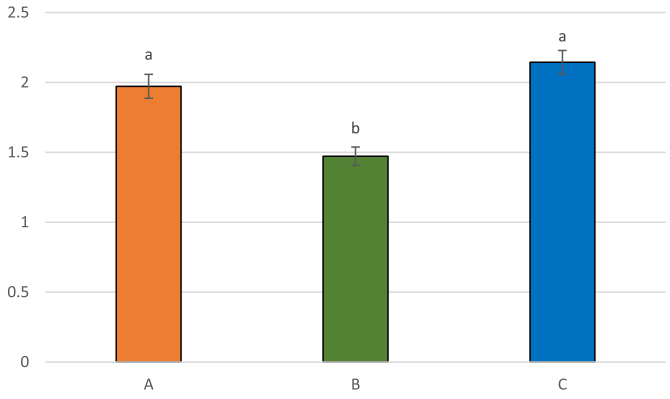

Fig. 2. Mean amount of DNA by treatment in ng/µL. (A) is the experimental extraction method; (B) is a standard CTAB extraction method; and (C) is a commercial kit extraction method. Each bar represents the mean of five DNA extractions of a single grape berry replicated five times, for a total of 25 extractions per treatment. Differing letters above the bars indicate significance at a *P* value of <0.05 according to Tukey's HSD.

To understand how this DNA extraction procedure may be impacting the physical surface of the grape berry, we conducted three extractions of supermarket-purchased *Vitis vinifera* cv. Flame Seedless grape berries using a negative control protocol (just TE buffer, no NaCl or SDS) and three with the previously described extraction buffer, and used a scanning electron microscope (SEM) to observe the changes in the grape surface. The samples were vortexed for 5 s in their respective buffers, then the skin of the berries was removed and cut into 0.5 cm-diameter fragments. To prepare samples for the SEM, we fixed the fragments in 3% glutaraldehyde, then placed in a buffered phosphate solution, and then in a post-fixation of 2% osmium. We then conducted two rinses in the phosphate buffer. We immersed the samples for one hour in each of 25%, 45%, 70%, 95% and 100% ethanol, followed by critical point drying and sputter coating.

3. Results

3.1. PCR results

The 150-bp bacterial amplicon and the 280-bp fungal amplicons were observed in all replicates using each of the DNA extraction techniques (Fig. 1), meaning that each of the methods were successful in amplifying DNA off the grape berry surface. The amount of DNA retrieved similar in our method was not significantly different than that of the MoBio

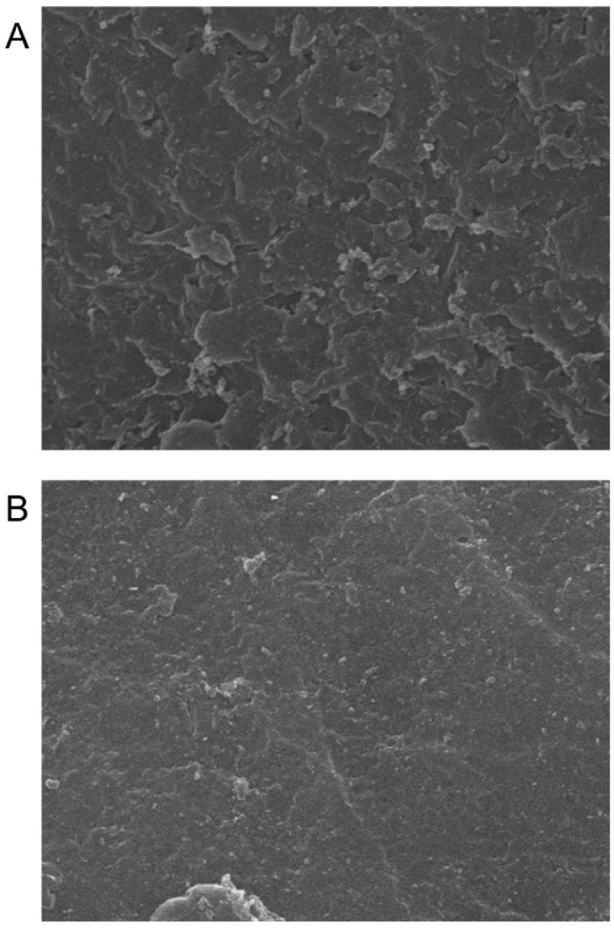

Fig. 3. Scanning Electron Microscope photos showing two 0.5 cm-diameter pieces of grape berry skin from supermarket-purchased *Vitis vinifera* cv. Flame Seedless grape berries. **(A)** A grape berry was placed in a 50 mL Falcon tube with Tris-EDTA buffer and vortexed for 5 s at room temperature, after which the berry skin was removed and cut into 0.5 cm-diameter pieces. The skin fragments were then fixed in 3% glutaraldehyde and then observed with the SEM. **(B)** A grape berry was placed in a 50 mL Falcon tube with Tris-EDTA+NaCl buffer solution plus 10% SDS and vortexed for 5 s at room temperature, after which the berry skin was removed and cut into 0.5 cm-diameter pieces. The skin fragments were then fixed in 3% glutaraldehyde and then observed with the SEM.

PowerSoil Kit commercial kit and both were higher than that of the CTAB extraction (Fig. 2). Per 10 samples, our method took approximately 50–60 minutes, while the MoBio PowerSoil Kit took 30–40 and the CTAB extraction took between 2.5 and 3 hours.

3.2. Microscopy results

The SEM photos reveal an intact waxy cuticle on those fragments in which the TE buffer was used alone (Fig. 3A), whereas there is a disruption in the waxy cuticle in those in which the NaCl and SDS was used in the extraction (Fig. 3B).

4. Discussion

This protocol was used in both Sanger sequencing and Illumina sequencing studies [13], yielding fungal and bacterial data. In one study, microbes were isolated from the surface of pea-sized grape berries, an early phenological time point at which the abundance of microbes on the surface was very low, and again at four later phenological time points, so as to demonstrate the season-wide microbial changes.

Because we did not have trouble with PCR amplification nor with sequencing, we continued using the protocol. While there may be options for DNA extraction that provide higher-quality DNA, our method of extraction is advantageous in three respects. (i) The reduced number of steps should translate into increased yield, important for small biomass samples, though admittedly at the cost of quality; (ii) The DNA extraction begins in the field, by cutting the berries directly into a tube containing the extraction buffer, and the agitation that it undergoes during transport back to the lab aids in removing microbes from the berry surface; (iii) the cost of materials required for the extraction procedure is very low in comparison to the cost of commercial kits; and (iv), all of the solutions required for the procedure have low toxicity, unlike the phenyl-chloroform extraction in which part of the extraction procedure takes place in a fume hood (Table 1). While we have used this technique solely on grape berries, these three components allow this DNA extraction method to be widely applicable in applied scientific research that involves field sampling. Instead of risking the manipulation of surface microbial communities by placing the sample in a bag and transporting it to the lab, during which the sample is subjected to temperature and/or humidity differences, as well as variable incubation periods, this technique allows the extraction to start as soon as the sample is removed from the plant. Moreover, it is not only useful for samples in which the microbes are difficult to remove from the surface, it also works well for DNA extractions of pure isolates in the lab [13].

Due to the increasing prevalence of microbiome studies, it is important to develop new techniques that address the challenges of certain matrices, like the grape berry surface. Techniques such as this one, which not only successfully extract the microbes from the grape surface, but does so in a safe, inexpensive, high-yielding and expeditious fashion could allow for increased accessibility of microbial studies on many different plant surfaces that were previously determined to have limited microbial populations.

Table 1
Comparison of toxicity levels of reagents used in our method of DNA extraction, the MoBio PowerSoil Kit and with a CTAB extraction. All toxicity levels were acquired from Safety Data Sheets (SDS). LD50 Dermal toxicity levels are reported on rabbits and LD50 Oral and Inhalation toxicity levels are reported on rats.

mg/kg	Phenol	Chloroform	Ethanol	Isopropanol
LD50 Dermal	630	>20000*	16000	12800
LD50 Oral	317	695	7060	5045
LC50 Inhalation	8h 900 mg/m^3	47702 mg/m^3	4h 117–125 mg/l	4h 37.5 mg/l
Our method			x	x
MoBio PowerSoil Kit			x	x
CTAB	x	x	x	x

Declarations

Author contribution statement

M. E. Hall: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

L. Cadle-Davidson: Conceived and designed the experiments.

W. F. Wilcox: Conceived and designed the experiments.

Contributed reagents, materials, analysis tools or data. Z. Fang: Performed the experiments.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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