Comparison of Tissue-Disruption Methods for PCR-Based Detection of Plant Pathogens

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Abstract

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Polymerase chain reaction-based detection of plant-associated microbes depends on physical disruption of tissues of the host and microorganism in order to liberate nucleic acids during extraction. Using six types of plant tissues as well as an oospore preparation of *Phytophthora capsici*, we evaluated the use of pressure-cycling technology (PCT) compared with several common techniques for physical tissue disruption. With all tissues tested, bead-beating provided excellent yields of amplifiable nucleic acid, with a few

inconsistent exceptions. The use of PCT did not consistently improve nucleic acid yields or “amplifiability”. The use of a mortar and pestle to physically disrupt plant tissue also provided good results at low cost, though it was not consistently as effective as the bead-beater. Furthermore, handling of ground tissues in an open mortar may present more challenges in minimizing cross-contamination than working with tissues pulverized in a bead-beater tube.

The practical importance of nucleic acid-based detection of plant pathogens has grown steadily during the past several decades. The ever-expanding suite of such tools has strengthened the science and practice of plant diagnostics (11,16), and these tools form a significant and growing component of laboratory practices used throughout the National Plant Diagnostic Network (14).

Current protocols for nucleic acid-based detection of plant-infecting microorganisms usually include some sort of tissue-disruption step as part of nucleic acid extraction. The physical rupture of cell walls releases protoplasmic contents, making nucleic acids available for purification and concentration. In most instances, physical disruption and lysis is accomplished through the use of mortars and pestles, bead-beaters, and similar tools that pulverize the sample tissues. Recently, several laboratories evaluated the use of an alternative approach to liberating nucleic acid from plant tissues: pressure-cycling technology (PCT). PCT differs significantly from other sample-preparation methods, in that it lyses tissues and liberates nucleic acids by subjecting samples to alternating cycles of ambient and high pressure (241 MPa = 35,000 psi).

Okubara et al. (8) found that PCT provided good yields of polymerase chain reaction (PCR)-amplifiable DNA of *Rhizoctonia solani* AG-8 and *Pythium abopressorium* from lyophilized wheat roots, and that it was superior to bead-beating for extracting DNA of these pathogens in soil samples. They also found that the DNA obtained via PCT exhibited less shearing than that obtained via bead-beating. In a preliminary report, Schaad et al. (13) also reported favorable results using PCT technology for detection of ‘*Candidatus Liberibacter*’ species in citrus. Based on these results, we hypothesized that PCT would be useful for routine, PCR-based diagnostic detection of pathogens associated with plant tissues. However, additional studies over a wider range of pathogens and tissue types seemed appropriate before diagnostic laboratories invested heavily in this new technology.

The objective of this study was to compare DNA yields and “amplifiability” of nucleic acids obtained from plant tissues and oospore preparations via PCT against those obtained by bead-beater and other techniques of mechanical tissue disruption.

Materials and Methods

Biological materials. All naturally infected tissues were collected in central Kentucky. Petioles of red oak naturally infected with *Xylella fastidiosa* and in a naturally air-dried state upon collection were placed into a mesh bag (item number ACC 00930/0100; AgDia Inc.), pounded with a shop hammer, and chopped into pieces 2 to 3 mm in size. Japanese walnut leaflets naturally infected with phytoplasma were stored, air dried at room temperature, and chopped into small fragments before processing. (Samples were processed in a dried state if processing was not possible soon after collection.) Corn leaves with sori resulting from natural infection by *Puccinia polysora* were pressed in a phone book and allowed to dry at room temperature for several days. These were cut into 6-by-6-mm squares and stored at 5°C before processing. Freshly collected leaves of cultivated, field-grown grape were surface-sterilized in 0.04% NaOCl for 5 min, followed by several rinses in sterile water and air drying. Leaves were sprayed to runoff on the adaxial side with *Brettanomyces bruxellensis* (ATCC number 34448) at 2.3×10^5 cells/ml suspended in sterile water; leaves were then air-dried to allow evaporation of surface moisture. Roots of 11-day-old greenhouse-grown squash plants were inoculated with agar plugs containing mycelium and sporangia of *Phytophthora capsici*, flooded for 4 days to promote infection, and watered as needed for 4 days. Roots were harvested by washing in tap water, chopped into approximately 1-cm lengths, air-dried overnight in a hood to allow evaporation of surface moisture, and stored at -20°C in petri dishes. Leaves of greenhouse-grown tobacco plants mechanically inoculated at the four-leaf stage with *Tobacco etch virus* (TEV) were harvested 7 to 10 days postinoculation and processed as fresh tissue. In some infected plant tissues, oomycete infection may have progressed to the point where the pathogen is present primarily as oospores resistant to DNA extraction. In order to compare methodologies for disruption of these survival structures, oospores of *P. capsici* were produced in vitro based on modifications of procedures described previously (1,5).

All tissue manipulations were made with attention to preventing cross-contamination (sterilized dissection tools and so on). Within each experiment, all pieces of a tissue type were pooled and mixed before dispensing into experimental units for tissue disruption.

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*The e-Xtra logo stands for “electronic extra” and indicates that four supplementary figures are available online.

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Tissue disruption methods. Samples of infected plant tissues representing the six pathosystems described above were individually weighed (100 mg and 15 mg for tissues that were fresh or dried, respectively; slight deviations from these target weights in individual samples were noted and used to correct results to standardized weights). Weighed samples were subjected to the following treatments in order to disrupt tissues. For all tissue samples, immediately after tissue disruption and addition of extraction buffer, sample tubes were placed on ice until all samples in the experiment were processed.

Mortar and pestle method. Liquid nitrogen was added to a mortar and allowed to partially evaporate until the mortar and pestle were well-cooled. Sample tissue was added, ground to a fine powder, and transferred to a labeled, sterile, 1.5-ml microcentrifuge tube. Extraction buffer (1.2 ml) was added and vortexed, and tubes were placed on ice. Extraction buffers used are described below.

Bead-beater alone. Sample tissue was added to a sterile 2-ml bead-beater tube (catalog number 72.693; Sarstedt) containing two 6.0-mm sterile glass beads; 100 mg of autoclaved quartz sand was also included in tubes containing squash roots or oak petioles. Tubes were immediately sealed and flash-frozen in liquid nitrogen, and tissue was pulverized using a Mini-BeadBeater-1, (Biospec Products 3110BX) operating at 2,500 rpm for 30 s (4,200 rpm for 40 s for oak petioles). The flash-freeze/tissue-disruption step was repeated up to two times if the tissue did not visually appear to be pulverized into a fine powder. Extraction buffer was immediately added as described above, the homogenate was vortexed, and samples were immediately placed on ice.

Bead-beater plus pressure cycling. Tissue was disrupted as in the bead-beater treatment and was followed by pressure cycling. After bead-beating, 1.2 ml of modified cetyltrimethylammonium bromide (CTAB) buffer was added, tubes were vortexed, glass beads were removed aseptically, and the homogenate was transferred into the cap-end of a Pressure BioSciences PCT Shredder PULSE tube (part number FT00500-SR) and subjected to pressure cycling using a Pressure BioSciences Barocycler, with 20 cycles of alternating conditions of 241 MPa (= 35,000 psi) for 20 s followed by ambient pressure for 20 s. After pressure cycling, the homogenate was aseptically transferred to a sterile 1.5-ml microcentrifuge tube, vortexed, and placed immediately on ice.

PCT Shredder Kit plus pressure cycling. Sample tissue was placed into a Shredder PULSE tube and ground for 30 s using the Pressure Biosciences PCT Shredder Driver and Holder, and this step was repeated once. (For two tissue types that were difficult to grind in the Shredder PULSE tube [oak petioles and squash roots], 100 mg of autoclaved granite grit was added into the “ram” end of the each Shredder PULSE tube in order to facilitate tissue disruption.) CTAB extraction buffer (1.2 ml) was added, tubes were capped and placed into the Barocycler, and pressure cycling was conducted as described above. The homogenate was handled as described previously.

In vitro preparations of oospores of *P. capsici* were tested separately from tests with infected plant tissues, because results from

the latter may reflect detection of DNA from mycelia rather than from oospores refractory to mechanical breakage. Oospore preparations were subjected to modified tissue-disruption methods. The bead-beater treatment consisted of adding approximately 100,000 oospores (previously pelleted, supernatant drawn off, and frozen) to a bead-beater tube half-filled with sterile, 0.5-mm zirconium silica beads (catalog number 11079105z; BioSpec Products), bead-beating the pellet at 4,200 rpm for 40 s without buffer, then adding 1.4 ml of CTAB buffer. For the treatment involving bead-beating plus pressure cycling, oospore pellets were treated as in the bead-beater treatment; buffer containing disrupted oospores was transferred to a Shredder PULSE tube, and pressure cycling was conducted as for disrupted plant tissue. The treatment consisting of pressure cycling only involved transferring frozen, dried oospores to a Shredder PULSE tube, adding 1.4 ml of buffer (because pressure cycling requires the presence of liquid), and pressure cycling as with disrupted plant tissue. To test the effect of sonication on tissue disruption, 0.2 ml of CTAB buffer was added to dried pellet of oospores and sonication was applied three times in 5-s pulses using a Tekmar sonicator set at an amplitude of 75; the remaining 1.2 ml of extraction buffer was added and contents were transferred to a Shredder PULSE tube and subjected to pressure cycling. Following tissue disruption, contents of all processed oospore samples were transferred to sterile 1.5-ml microcentrifuge tubes, vortexed, and placed immediately on ice. Oospore suspensions were not subjected to treatments using the Shredder PULSE tube alone or using the mortar and pestle, because these techniques did not seem suitable for disruption of aqueous suspensions of microscopic spores.

For each experiment, one tissue sample (= one biological replicate) in each tissue-disruption treatment was disrupted before commencing with the next replicate set of tissue samples in the experiment, so that each replication could be considered a “block” in a randomized complete block design. Once all four replicates of the experiment were completed, DNA was extracted using a modified CTAB method (6), and RNA was extracted using the RNeasy Plant Mini Kit (catalog number 74904), with an extraction buffer containing 0.2% 2-mercaptoethanol. Extracted nucleic acids were stored at -20°C until use. Each tissue-disruption experiment was performed three times in separate experiments.

DNA quantification and PCR analyses. Nucleic acid extracts were quantified using an Invitrogen Qubit fluorometer (Life Technologies). Nucleic acid yields were expressed as nanograms of sample DNA or RNA per 100,000 oospores, 15 mg corn leaf tissue, or 100 mg of all other sample tissue types.

One aliquot of DNA from each replicate sample extract was amplified via PCR (= one technical replicate per sample DNA extract). Real-time PCR and conventional PCR were performed in a Cepheid SmartCycler and an Applied Biosystems GeneAmp PCR System 9700, respectively. Key PCR conditions are described in Table 1. For real-time PCR assays, 10 ng of sample DNA (or 20 ng, in the case of corn leaves) was added to the reaction tube (final reaction volume = 25 µl). For assays run as real-time PCR protocols, cycle threshold (C_t) values were compared among treatments.

Table 1. Key polymerase chain reaction (PCR) conditions used for testing extracts obtained via different tissue-disruption techniques

Source of sample	Primers/probes used	Thermocycling conditions ^y	Reference
Red oak petioles	XfF1, XfR1, XfP1	95°C for 10 min, 40 cycles of 95°C for 1 s, and 60°C for 20 s	12
Japanese walnut leaflets	R16mF2, R16mR1	94°C for 2 min, 30 cycles of 94°C for 1 min, 58°C for 2 min, and 72°C for 3 min	4
Corn leaves	PpolF, PpolR, PPOLY	95°C for 120 s, 40 cycles of 95°C for 5 s, 60°C for 30 s, and 72°C for 1 s	2
Grape leaves	DBRUXF, DBRUXR	95°C for 5 min, 45 cycles of 95°C for 5 s, and 69°C for 30 s	10
Squash roots and oospores	Pcap-q-1F, Pcap-q-1R, MGB probe	95°C for 10 min, 40 cycles of 95°C for 15 s, and 60°C for 60 s	9
Tobacco leaves	5'-ACACCAATCCTCACACCGGAT-3' 5'-GACCATGAGTGTGTGTGTC-3'	95°C for 30 s, 45 cycles of 95°C for 3 s, and 60°C for 30 s	None ^z

^y PCR reaction mixtures varied somewhat from those reported in source literature but were validated in all tests using appropriate positive controls (DNA of known origin) and negative controls (sterile molecular-grade water).

^z Designed by authors.

For Japanese walnut infected with phytoplasma, the lack of a suitable real-time PCR protocol necessitated the use of conventional PCR to amplify the target (round-one primers of Gundersen and Lee [4]; Table 1). PCR was conducted on 10-ng aliquots of sample DNA in a 50- μ l volume. After PCR of walnut extracts, overall DNA yield following PCR was estimated by first confirming the presence of only the target band via agarose electrophoresis, cleaning the amplicon using the Qiagen MinElute PCR Purification Kit (number 28004), and estimating amplicon yield using the Qubit fluorometer.

For TEV, cDNA was produced from RNA extracts using the Sensiscript RT reverse-transcriptase kit (catalog number 205211; Qiagen) and quantified. The cDNA was diluted 100-fold in sterile molecular-grade water, and a 1- μ l aliquot was used for the PCR reaction. Primers were designed to target the N1b protein of TEV (Table 1), which yielded a 105-bp amplicon. Following PCR, this product was readily detected in infected plants by agarose electrophoresis as well as by melt-curve analysis of reaction mixtures which included SYBR-Green. No amplicon was detected by either method when extracts from uninfected plants were tested.

Data analysis. The Shapiro-Wilk test of normality was used to test the assumption of normality in the datasets generated (SAS version 9.3; SAS Institute Inc.). With the exception of oospore data, few sample populations departed from normality ($P > 0.05$) and, therefore, were analyzed without transformation. Data for DNA concentration and amplifiability from oospore extracts were $\log_{10}$ -transformed to improve the fit to the assumption of normality. As tissue disruption was performed in each tissue-disruption treatment being tested before commencing with the next replicate set of treatments, data were subjected to analysis of variance following a randomized complete block design using PROC GLM of SAS, with “replicate” as the blocking factor and with each trial being analyzed as a separate experiment. Means were compared using the Waller-Duncan k -ratio t test ($k = 100$, $P \approx 0.05$).

Results

DNA yields. In at least two of three experiments with each plant tissue tested, the bead-beater without pressure cycling provided nucleic acid yields as high or higher than ($P = 0.05$) other treatments tested (Tables 2–7). Only two exceptions occurred: one ex-

periment using petioles of red oak (Table 3) and one using walnut leaves (Table 7). When examined microscopically, tissue disrupted via bead-beating was highly pulverized, especially when using air-dried host tissues (Supplementary Figures S1–S3). Surprisingly, adding a pressure cycling step following tissue disruption using the bead-beater offered no consistent advantage in nucleic acid yields over the bead-beater alone in all tissues tested (Tables 2–8).

Using oospore preparations, the bead-beater also performed as well as other tissue-disruption treatments in two trials (Table 8). In the third, sonication plus pressure cycling provided significantly ($P < 0.05$) and substantially higher DNA yields than all other treatments; in that trial, we also observed a high frequency of oospore breakage only in the sonication plus PCT treatment (Supplementary Figure S4).

With fresh leaves of grape and tobacco and with dried leaves of corn, the mortar and pestle generally provided yields statistically equivalent to the bead-beater (Tables 4–6). However, with fibrous or hard tissues (dried squash roots, dried red oak petioles, and mature, dried leaves of Japanese walnut), the mortar and pestle provided less ($P = 0.05$) nucleic acid than the bead-beater, although what was harvested was amplifiable (Tables 2, 3, and 7). Similar results were obtained using the Shredder PULSE tube. When examined microscopically, neither provided the degree of pulverization obtained using the bead-beater, although tissue ground via mortar and pestle visually appeared to be more fragmented than that treated with the PCT Shredder PULSE tube.

Significant—although not extreme—variability was observed in DNA yields obtained in all tissues tested, with coefficients of variation usually in the range of 20 to 100%.

PCR results. Amplifiability of extracted DNA was evaluated by comparing C_t values or amplicon yield among treatments, given that all PCR reaction tubes in an experiment received the same initial quantity of sample DNA. By this standard, the bead-beater alone, and the bead-beater plus pressure cycling, performed relatively similarly (Tables 2–8). One or both treatments were almost always statistically equivalent to the best treatment in each test ($P = 0.05$) and, when one of these two methods outperformed the other, the difference was modest and did not occur consistently (Tables 2 and 3).

Sample DNA obtained via the Shredder PULSE tube plus pressure cycling always produced successful amplification but fre-

Table 2. Evaluation of four tissue-disruption methods for yield of sample DNA and polymerase chain reaction cycle threshold (C_t) values using air-dried squash roots infected with *Phytophthora capsici*^x

Method	Frozen ^y	Cycling ^z	DNA yield (ng of DNA/100 mg of tissue)			C_t values		
			Trial 1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 3
Bead-beater	Yes	No	12,400 a	20,700 a	54,700 a	18.8 a	21.9 ab	19.9 b
Bead-beater	Yes	Yes	14,100 a	24,800 a	41,800 ab	19.2 a	23.3 c	18.7 a
Mortar and pestle	Yes	No	5,450 b	6,740 b	20,100 bc	23.3 b	22.7 bc	19.5 b
Shredder PULSE tube	No	Yes	2,700 b	2,500 b	8,910 c	22.5 b	21.2 a	21.5 c
CV (%)	...	...	44.0	21.1	45.6	7.4	2.9	1.9

^x Within each trial, means followed by the same letter are not significantly different (Waller-Duncan k -ratio t test, $k = 100$, $P \approx 0.05$). CV = coefficient of variation.

^y Tissue flash-frozen in liquid nitrogen before bead-beating; mortar and pestle prechilled in liquid nitrogen.

^z Pressure cycling.

Table 3. Evaluation of four tissue-disruption methods for yield of sample DNA and polymerase chain reaction cycle threshold (C_t) values air-dried red oak petioles naturally infected with *Xylella fastidiosa*^x

Method	Frozen ^y	Cycling ^z	DNA yield (ng of DNA/100 mg of tissue)			C_t values		
			Trial 1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 3
Bead-beater	Yes	No	1,300 a	4,400 a	269 b	20.0 a	18.8 a	19.9 b
Bead-beater	Yes	Yes	762 b	3,440 b	276 b	19.2 a	19.0 a	19.4 a
Mortar and pestle	Yes	No	398 bc	782 c	867 a	20.1 a	19.4 a	19.1 a
Shredder PULSE tube	No	Yes	84 c	131 d	146 c	23.0 b	19.7 a	19.9 b
CV (%)	...	...	42.3	19.9	8.9	3.8	3.8	1.5

^x Within each trial, means followed by the same letter are not significantly different (Waller-Duncan k -ratio t test, $k = 100$, $P \approx 0.05$). CV = coefficient of variation.

^y Tissue flash-frozen in liquid nitrogen before bead-beating; mortar and pestle prechilled in liquid nitrogen.

^z Pressure cycling.

quently with reduced ($P = 0.05$) efficiency compared with other treatments (Tables 2, 3, 6, and 7). In one test using oospores of *P. capsici*, DNA extracted via sonicator produced better amplifiability than the other methods tested; no difference ($P > 0.05$) was observed among methods in the other two tests (Table 8).

With the exception of DNA obtained from Japanese walnut, variability in PCR amplifiability (as measured by C_t values) was low, with coefficients of variation $\leq 10\%$ in almost all cases.

Discussion

In our tests, the best overall method for mechanical disruption of varied plant tissues for PCR analysis was the bead-beater. We base this on the fact that yield and amplifiability of extracted nucleic acids were high using this device, with few, inconsistent exceptions. Bead-beating followed by pressure cycling added approximately 13 min to the extraction process without consistently improving yield or amplifiability. Therefore, we concluded that the high cost of the pressure-cycling unit was not a justifiable expense when extracting nucleic acid for PCR-based detection of plant pathogens, at least for the tissues examined in this study. We also concluded that the use of the Shredder PULSE tubes for mechanical disruption of fibrous or hard tissues was substantially less effective than the bead-beater.

Previous studies suggested that PCT might be advantageous for DNA extraction from plant tissues. However, methodological differences may account for differences between results of previous studies and our own. Schaad et al. (13) reported good results using PCT in preliminary studies of '*Ca. Liberibacter*' detection using PCT combined with the PCT Shredder PULSE tube. However, different DNA extraction kits were used for different tissue-disruption methods, making direct comparisons difficult. In our studies, we used the same post-disruption DNA extraction procedure for all treatments in all experiments, permitting direct comparison of the tissue-disruption methods. Okubara et al. (8) reported obtaining substantially higher DNA yields from pressure cycling versus bead-beating when extracting from soil samples; in our studies, we only worked with plant tissues. In this earlier research, only one plant tissue was evaluated: lyophilized wheat roots. This tissue was reported to be "rubbery" and recalcitrant to pulverization using a mortar and pestle or bead-beater, whereas the researchers reported good DNA yields using PCT. Although it is possible that PCT will provide an advantage with certain unusual types of samples of plant tissue, such as reported in that study, none of the plant tissues we tested exhibited substantial recalcitrance to pulverization using a bead-beater, even rather hard tissues like dried petioles of red oak. We consistently were able to obtain

Table 4. Evaluation of four tissue-disruption methods for yield of sample DNA and polymerase chain reaction cycle threshold (C_t) values using dried corn leaves naturally infected with *Puccinia polysora*^x

Method	Frozen ^y	Cycling ^z	DNA yield (ng of DNA/15 mg of tissue)			C_t values		
			Trial 1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 3
Bead-beater	Yes	No	32.9 a	24.4 a	48.6 a	21.2 a	18.5 a	20.1 a
Bead-beater	Yes	Yes	31.3 ab	25.9 a	23.8 ab	19.6 a	20.0 a	20.7 a
Mortar and pestle	Yes	No	12.3 bc	12.2 ab	21.4 ab	21.2 a	20.0 a	20.0 a
Shredder PULSE tube	No	Yes	5.4 c	1.5 b	4.2 b	20.7 a	20.9 a	22.1 a
CV (%)	...	...	57.2	76.6	98.6	4.5	7.3	10.2

^x Within each trial, means followed by the same letter are not significantly different (Waller-Duncan k -ratio t test, $k = 100$, $P \approx 0.05$). CV = coefficient of variation.

^y Tissue flash-frozen in liquid nitrogen before bead-beating; mortar and pestle prechilled in liquid nitrogen.

^z Pressure cycling.

Table 5. Evaluation of four tissue-disruption methods for yield of sample DNA and polymerase chain reaction cycle threshold (C_t) values using fresh grape leaves superficially colonized by *Dekkera bruxellensis*^x

Method	Frozen ^y	Cycling ^z	DNA yield (ng of DNA/100 mg of tissue)			C_t values		
			Trial 1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 3
Bead-beater	Yes	No	1,670 a	1,260 a	911 ab	26.4 a	25.4 a	25.3 a
Bead-beater	Yes	Yes	1,620 a	1,380 a	710 ab	25.8 a	26.0 a	24.8 a
Mortar and pestle	Yes	No	897 a	802 a	1,100 a	25.2 a	25.9 a	24.5 a
Shredder PULSE tube	No	Yes	436 a	491 a	403 b	24.4 a	25.9 a	23.3 a
CV (%)	...	...	113	63	41	6.5	5.2	8.8

^x Within each trial, means followed by the same letter are not significantly different (Waller-Duncan k -ratio t test, $k = 100$, $P \approx 0.05$). CV = coefficient of variation.

^y Tissue flash-frozen in liquid nitrogen before bead-beating; mortar and pestle prechilled in liquid nitrogen.

^z Pressure cycling.

Table 6. Evaluation of four tissue-disruption methods for yield of sample RNA yield, cDNA following reverse transcription, and polymerase chain reaction cycle threshold (C_t) values using fresh tobacco leaves infected with *Tobacco etch virus*^x

Method	Frozen ^y	Cycling ^z	RNA yield (ng RNA/100 mg tissue)			cDNA yield (ng of TEV cDNA obtained from 48 ng of RNA extract)			C_t values obtained using 1 μ l of 1/100 dilution of cDNA		
			Trial 1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 3
Bead-beater	Yes	No	76.3 a	43.0 ab	61.0 a	15.3 a	24.2 a	14.7 a	26.1 a	24.0 a	24.5 ab
Bead-beater	Yes	Yes	83.1 a	56.2 ab	53.1 a	15.1 a	24.1 a	14.9 a	26.2 a	24.9 a	23.6 a
Mortar and pestle	Yes	No	68.5 a	65.2 a	33.9 a	15.8 a	15.8 b	15.0 a	27.3 a	24.8 a	25.2 b
Shredder PULSE tube	No	Yes	91.7 a	32.6 b	39.0 a	15.3 a	18.6 ab	13.8 a	26.0 a	27.3 b	24.7 ab
CV (%)	...	...	16.5	28.7	37.5	14.9	21.6	15.2	3.4	2.6	3.4

^x Within each trial, means followed by the same letter are not significantly different (Waller-Duncan k -ratio t test, $k = 100$, $P \approx 0.05$). CV = coefficient of variation.

^y Tissue flash-frozen in liquid nitrogen before bead-beating; mortar and pestle prechilled in liquid nitrogen.

^z Pressure cycling.

suitable quantities of nucleic acids for PCR testing or reverse transcription using a bead-beater, with no consistent incremental benefit from PCT. One difference worth noting between the prior study (8) and our own was that the pressure-cycling extraction medium in the prior work included garnet grit placed in the cap end of the Shredder PULSE tube. It seems possible that inclusion of garnet grit in the cap end of the Shredder PULSE tube during pressure cycling may improve tissue disruption by facilitating physical abrasion or disruption of sample tissue during the process of cycling from extreme (235 MPa) to ambient pressure. In our studies, however, bead-beating consistently provided a high degree of physical pulverization; therefore, we question whether PCT-based extraction from plant tissues could be improved over bead-beating tissues flash-frozen in liquid nitrogen. An additional concern with PCT-based nucleic acid extraction is that, although it may have the potential to improve release of nucleic acids from certain tissue fragments, the current technology is not suited to flash-freezing of sample tissues. When using bead-beating, we froze tissue in liquid nitrogen before each pulverization, and then we maintained the pulverized tissue in liquid nitrogen until extraction buffer was added. In contrast, during pressure cycling, the sample can be chilled but cannot be frozen, providing an opportunity for enzymatic degradation of nucleic acids during tissue lysis.

The mortar and pestle was an acceptable method for obtaining amplifiable DNA, though not as effective as the bead-beater. The mortar and pestle provided nucleic acid yields equal to the bead-beater using easily disrupted tissues but provided reduced yields using fibrous or hard tissues, sometimes yielding as little as approximately 20 to 30% of the nucleic acid obtained using the bead-beater. There appeared to be no problem with quality of the extracted nucleic acid because, in most tests, the nucleic acid obtained was as suitable for PCR and reverse transcription as that obtained using the bead-beater. However, additional limitations of the mortar and pestle included difficulty in transferring all macerated tissue to microfuge tubes for DNA extraction, longer processing time compared with the bead-beater (though not as long as pressure cycling), and the potential for laboratory contamination with sample DNA during sample grinding or transfer of lysate. We concluded that the mortar and pestle is suitable for circumstances

where funds limit the purchase of a bead-beater plus tubes but that the bead-beater would be preferable in most diagnostic laboratories.

For DNA extraction from oospores, the sonicator plus PCT outperformed the other tissue-disruption methods in one of three trials for both measures evaluated (DNA yield and PCR amplifiability) and was equivalent in the other two trials. In the trial where higher DNA yield was observed, the amount obtained was nearly an order of magnitude higher than the other methods. Regarding amplifiability, in the single trial where C_t values obtained using DNA extracted via sonication were statistically lower than the bead-beater, the difference observed—approximately two cycles—was modest. Taken as a whole, these results suggest that, for diagnostic laboratories, acceptable extracts are likely to be obtained from oospores using a bead-beater, especially because oospore-laden plant tissues usually will also contain some mycelium as well, and we successfully used bead-beating to extract DNA from infected squash roots that likely contained mostly mycelium and few mature oospores. However, research programs that work regularly with oospore populations in environmental sources may wish to evaluate sonication for DNA extraction.

Sample collection and processing represent significant and ongoing challenges for PCR testing of microbial agents in their hosts or the environment (16), and that was reflected in our results. Although all methods for nucleic acid extraction were well standardized, in most experiments, significant—though not extreme—variability was observed in yields of extracted nucleic acid. This was reflected in values for coefficients of variation (CVs) as well as in trial-to-trial variability (the latter being substantial in cases such as Tables 2 and 3). With respect to CV values observed for total nucleic acid extracted, although these values do not seem excessive in our estimation, they do represent experimental error (in a statistical sense) during nucleic acid extraction. With respect to trial-to-trial variability in total nucleic acid extracted, each trial was conducted as a completely separate, independent experiment. Thus, trial-to-trial variation may be due, in part, to mechanical or biochemical differences among the pools of tissue fragments used in each experiment. Because our nucleic acid extraction procedures were highly standardized within and between experiments, meth-

Table 7. Evaluation of four tissue-disruption methods for yield of sample DNA following extraction and yield of amplicon DNA following a single-round conventional polymerase chain reaction using air-dried leaves of Japanese walnut naturally infected with an undetermined phytoplasma^x

Method	Frozen ^y	Cycling ^z	DNA yield (ng of DNA/100 mg of tissue)			Amplicon yield (pg of DNA/reaction)		
			Trial 1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 3
Bead-beater	Yes	No	1,080 b	4,370 a	4,710 a	50.5 a	100 b	43.6 ab
Bead-beater	Yes	Yes	5,230 a	4,600 a	5,420 a	68.4 a	116 ab	33.9 b
Mortar and pestle	Yes	No	1,620 b	1,460 b	1,810 b	51.1 a	154 a	77.0 a
Shredder PULSE tube	No	Yes	252 c	567 c	1,180 b	48.4 a	22.8 c	12.8 b
CV (%)	...	...	21.3	13.6	25.1	57.5	27.5	58.6

^x Within each trial, means followed by the same letter are not significantly different (Waller-Duncan k -ratio t test, $k = 100$, $P = 0.05$). CV = coefficient of variation.

^y Tissue flash-frozen in liquid nitrogen before bead-beating; mortar and pestle prechilled in liquid nitrogen.

^z Pressure cycling.

Table 8. Evaluation of four tissue-disruption methods for yield of sample DNA and polymerase chain reaction cycle threshold (C_t) values using an aqueous suspension of oospores of *Phytophthora capsici*^w

Method	Frozen ^y	Cycling ^z	DNA yield (ng of DNA/10 ⁵ oospores) ^x			C_t values		
			Trial 1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 3
Bead-beater	Yes	No	7.70 a	2.72 a	5.97 b	22.7 a	20.9 b	19.4 a
Bead-beater	Yes	Yes	9.66 a	1.54 ab	6.03 bc	20.6 a	21.1 b	20.4 a
Sonicator	No	Yes	5.73 a	4.16 a	48.2 a	21.4 a	19.0 a	18.4 a
None	No	Yes	5.39 a	0.63 b	1.99 c	21.3 a	22.7 c	19.4 a
CV (%)	...	...	38.3	32.5	23.0	5.9	2.98	13.3

^w Within each trial, means followed by the same letter are not significantly different (Waller-Duncan k -ratio t test, $k = 100$, $P = 0.05$). CV = coefficient of variation.

^x Statistical analysis based on log₁₀-transformed data; back-transformed means are presented for ease of interpretation.

^y Oospore preparation frozen in liquid nitrogen before bead-beating.

^z Pressure cycling.

ods for reducing this variation were not readily apparent. In contrast to our results for nucleic acid extraction, variability in PCR amplifiability was low (with the exception of phytoplasma DNA obtained from Japanese walnut). It is well known that pathogens can vary in density or titer within host tissue (3,7,15). Because attention was paid to careful mixing of solutions during all procedures for nucleic acid extraction and PCR setup, it seems likely that spatial variability in pathogen distribution and density within the host tissue was a significant contributor to the variation observed.

Based on our results, we conclude that bead beating is an efficient method for obtaining nucleic acids suitable for PCR and reverse transcription from plant samples. Given the expense, additional processing time and lack of consistent improvement in DNA yield or amplifiability, the use of PCT in routine diagnostic settings does not seem to be advantageous.

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