

Research

Use of Sodium Hydroxide DNA Extraction Methods for Nested PCR Detection of *Bretziella fagacearum* in the Sapwood of Oak Species in Minnesota

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Abstract

Oak wilt caused by *Bretziella fagacearum* is an important disease of *Quercus* spp.; however, its diagnosis may be confused with damage resulting from other diseases, insects, or abiotic factors. Laboratory diagnosis is important in such situations and when disease control action is desired. PCR tests can provide accurate lab diagnosis within 2 days. Two variations of a simple DNA extraction protocol using sodium hydroxide (NaOH) were compared with that of the proprietary protocol of a commercially available kit (CK) for nested PCR to detect the pathogen in oak sapwood. High frequencies of pathogen detection (98 to 100%

of 48 branch segments assayed) were found for northern pin oak using the two NaOH-based and the CK methods. Detection rates were similar but lower for bur oak (ranged from 58 to 79%) and white oak (ranged from 54 to 71%) regardless of DNA extraction method. Using our alternative DNA extraction protocols may reduce total time and cost of *B. fagacearum* detection in PCR-based diagnosis and other downstream applications.

Keywords: *Bretziella fagacearum*, DNA extraction, oak wilt fungus, *Quercus*

Bretziella fagacearum (Bretz) Z. W. deBeer, Marinc., T. A. Duong & M. J. Wingf. (syn. *Ceratocystis fagacearum* (Bretz) J. Hunt) causes one of the most important diseases of oak (*Quercus* spp.) in the eastern United States and Texas. Oak wilt, the systemic vascular disease caused by the pathogen, has been reported in more than 825 counties in 24 states (Juzwik et al. 2011). Recently, the disease range has expanded to include New York, with new oak wilt centers documented in upstate New York since 2008 (Jensen-Tracy et al. 2009) and on Long Island in southeastern New York since 2016 (New York State Department of Environmental Conservation 2020). Significant portions of Michigan, Minnesota, Texas, and Wisconsin have experienced ongoing epidemics in recent decades, although disease suppression programs have also been underway in most of these states since the early 1990s (Juzwik et al. 2011). Oak wilt can dramatically alter both urban and natural ecosystems if left untreated (Appel 1995).

In red oak (*Quercus* Section Lobatae), the endoconidia of the fungus are carried relatively quickly in the sapstream through large-diameter, springwood vessels (i.e., the vessels in the early

part of the annual growth ring) from the site of pathogen introduction to the upper crown. Once infected, red oak may succumb to complete wilt in as few as 4 to 6 weeks. In contrast, with members of the white oak group (*Quercus* Section *Quercus*), the internal spread of *B. fagacearum* endoconidia is slower and infection compartmentalized due to host response. Once infected, bur oak may not experience complete crown wilt for 2 to 4 years while infected white oak may live for many years (Pokorny 2015).

Diagnosis of oak wilt is generally straightforward in red oak species, where field diagnosis is often possible for arborists and foresters familiar with the disease. Characteristics used for field diagnosis of the disease in red oak include bronzing or water-soaking appearance of the leaves, pattern of wilt progression in the tree crown, and pattern of disease spread on the affected land parcel. However, field diagnosis in red oak may be confused, in particular, with symptoms of bacterial leaf scorch caused by *Xylella fastidiosa* subsp. *multiplex* (Gould and Lashomb 2005) and the damage resulting from outbreaks of the two-lined chestnut borer (*Agilus bilineatus*) (Haack and Acciavatti 1992). Field diagnosis of oak wilt in white oak group species is more problematic and symptoms vary by species (Juzwik and Appel 2016). For example, affected branches in bur oak are generally scattered throughout the crown of an infected tree whereas affected white oak may exhibit only one or two wilting branches or wilt of a main fork. Leaf symptoms also are more irregular in white oak species. In the Upper Midwest, oak wilt in bur oak may be confused with symptoms caused by the bur oak blight pathogen *Tubakia iowenensis* (Harrington et al. 2012). The overall decline that occurs over multiple years in an oak-wilt-affected white oak may be confused with gradual decline attributable to a number of other biotic agents or to abiotic or human-caused damage.

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Thus, laboratory diagnosis is required for accurate diagnosis in many cases, particularly if disease control action is planned. Success in oak wilt management is greatest when control actions are taken soon after early detection and timely diagnosis occur (Juzwik et al. 2011).

Due to the spotty or discontinuous colonization pattern of the pathogen in white oak species, the University of Minnesota Plant Disease Clinic often assays two times the number of sapwood chips from bur and white oak samples compared with red oak ones when conducting traditional isolation assays (J. Flynn, personal communication). To maximize chances of successful detection and avoid false-negative results, arborists and others are advised to remove several symptomatic branches from a suspected tree and cut several segments from each branch for submission to a diagnostic laboratory (Pokorny 2015; Yang and Juzwik 2017).

Currently, laboratory diagnosis of oak wilt is based on results from standard pathogen isolation (Pokorny 2015) or molecular assays. Nested and real-time PCR protocols with *B. fagacearum*-specific primers were first developed by Wu et al. (2011). These protocols were recently modified and evaluated by Yang and Juzwik (2017). At least one commercial diagnostic laboratory also uses proprietary molecular procedures for processing suspect oak wilt samples (Research Associates Laboratory, Allen, TX, U.S.A.). Commonly used molecular tests (arguably, the current “gold standard”) involve extraction and purification of DNA followed by PCR amplification of the DNA using primers from a selected gene region. The PCR product is then visualized using gel electrophoresis protocols. The nested PCR protocol reported by Yang and Juzwik (2017) involves extraction of DNA from sapwood drill shavings taken from actively wilting branch samples or lower stem sections of completely wilted trees. The amplification step is a two-part process starting with the general fungal primer pair internal transcribed spacer (ITS)1F/ITS4 and followed by further amplification with species-specific primers (CF01 and CF02) developed by Wu et al. (2011). Resulting bands for *B. fagacearum* appear at approximately 280 bp on the gel. DNA extraction can be completed using a commercially available kit (CK) that contains a proprietary solution for PCR inhibitors in plant or soil extracts (QIAmp DNA Stool Kit; Qiagen, Venlo, The Netherlands). The total time required for sample processing (from obtaining drill shavings through gel visualization) is approximately 2 days. We estimate that the materials cost for using the CK for DNA extraction is U.S.\$6.47 per sample. Previously published methods involving use of a strong base (sodium hydroxide [NaOH]) to extract DNA from plant samples could potentially be used to modify the above-referenced nested PCR protocol to reduce both cost and time for processing samples in plant diagnostic laboratories. In a comparative study of rapid and dependable methods for extracting DNA from environmental samples, Osmundson et al. (2013) found that NaOH extraction protocols of Wang et al. (1993) worked well in terms of detection success rate, cost, simplicity, and speed. The NaOH methods, as described by Wang et al. (1993) and Xin et al. (2003), use NaOH to extract DNA from plant samples, then neutralize it with Tris buffer. The obtained DNA is then amplified using a typical PCR procedure with additives to suppress any PCR inhibitors carried from the extract. However, Osmundson et al. (2013) offered several cautions when using NaOH extraction, particularly for substrates with low amounts of the target fungus present or that are rich in lignin or humic acids.

The purpose of this research was to evaluate the potential for substituting an NaOH-based DNA extraction protocol for the

proprietary extraction protocol of a CK commonly used for nested PCR based diagnosis of oak wilt in the laboratory. An alternative method for DNA purification and concentration in addition to a previously published NaOH protocol also was developed for comparison. The main objective of this study was to compare detection rates of *B. fagacearum* in red and white oak species using the current standard molecular protocol (DNA extraction and nested PCR amplification using a CK) with those obtained using DNA extracted following two NaOH protocols and then subjected to nested PCR amplification using the same CK. The standard isolation protocol for *B. fagacearum* was included for further comparison. The second objective was to compare the amount of total DNA obtained by each extraction method.

Materials and Methods

Sampling sites and branch sampling protocols. In early September 2018, one location near Stacy, MN, in the Carlos Avery Wildlife Refuge, with actively wilting northern pin oak (*Quercus ellipsoidalis*) was selected for sampling. Three branches (3.0 to 7.0 cm in diameter) were cut from each of four trees exhibiting classic oak wilt symptoms (leaf discoloration and xylem staining in branches) per the protocol of Yang and Juzwik (2017). Nonsymptomatic branches from healthy northern pin oak were collected to use as controls. Four segments (approximately 30 cm long) were cut from each branch, placed in plastic bags, stored on ice during transport to the laboratory, and placed in cold storage (4°C).

Between mid-July and early September 2019, multiple white oak (*Q. alba*) trees with scattered branches exhibiting foliar symptoms typical of oak wilt and located in street and park settings were selected for sampling from seven trees on several sites in Eagan, Apple Valley, and Minneapolis, MN. Between mid-June late August 2020, bur oak (*Q. macrocarpa*) branches were obtained from six bur oak trees in Stacy, St. Paul, and Becker, MN. Nonsymptomatic branches from healthy bur and white oak were sampled to use as controls. Segments (approximately 30 cm long) were obtained from each harvested branch and handled and stored as described above.

Branch sample processing and standard isolation protocols. In the laboratory, the bark was carefully peeled from each segment with a sterile drawknife to reveal the presence of outer xylem staining. In a laminar flow hood, four or five small wood chips were excised and placed on two 100-mm-diameter Petri plates containing oak wilt identification agar (Barnett 1953) per Yang and Juzwik (2017) protocols. The agar plates were incubated for 14 days at room temperature (approximately 24°C) under ambient lighting and checked daily after 7 days for the presence of suspected colonies. Subcultures were made as necessary onto half-strength potato dextrose agar plates to obtain pure cultures. Resulting isolates of *B. fagacearum* were identified based on colony morphology, characteristic odor, and presence of endoconidia. Isolation was also attempted from branches cut from healthy oak trees. All branch samples from northern pin oak were processed within 7 days of collection, while samples from bur and white oak were processed within 12 days of branch harvest. Only those trees which produced *B. fagacearum*-positive cultures (four trees of each species) were used in the DNA extraction phase of the study.

At the same time that the above isolations were performed, drill shaving samples were collected for DNA-based assays. Shavings were obtained using previously published protocols (Yang and Juzwik 2017). Approximately 2 ml of shavings was placed in each

of two 2-ml microcentrifuge tubes per segment. When branches were too small for making drill shavings, thin strips of outer sapwood exhibiting staining characteristic of oak wilt infection were shaved and then cut into small pieces. All tubes were stored at -20°C until DNA was extracted.

DNA extraction. Two general approaches were used to extract DNA from the drill shaving samples: (i) the protocol of a CK (QIAmp DNA Stool Kit; Qiagen) and (ii) modified versions of published protocols using NaOH (Lemke et al. 2011; Xin et al. 2003).

For extractions using the kit, samples were processed as described by Yang and Juzwik (2017). In short, drill shavings (approximately 110 mg) were placed in a 2-ml microcentrifuge tube with two sterilized metal beads (4.5 mm in diameter). Lysis buffer (750 μl) from the kit was added and the sample was homogenized, then heated at 70°C for 5 min. Extraction of DNA was then completed according to the manufacturer's instructions for the kit (mixing with ethanol, spin filtering, washing, and eluting), except that that buffer quantities were reduced to 75%.

For extraction of "crude" DNA using NaOH, drill shavings (approximately 110 mg) were placed in a microcentrifuge tube and 1.0 ml of 0.5 N NaOH (buffer A as described by Xin et al. [2003]) was added to sufficiently immerse the shavings (proportion used was 1 ml of NaOH to 110 mg of wood shavings). Vigorous agitation with a vortex mixer was performed for 2 min, then several times for 2 to 3 s during 10 min of soaking in NaOH. The mixture was then centrifuged (13,000 rpm for 2 min) and the resulting supernatant was mixed with 100 mM Tris-HCl (pH 4.0) (buffer B as described by Xin et al. [2003]) at a ratio of 1 to 9 (supernatant to buffer). The DNA extract obtained in this manner is referred to as "NaOH crude."

To obtain a purified and concentrated DNA extract, the NaOH crude extract was subjected to a spin filter step that is similar to that used with the CK. Specifically, two volumes of the crude extract (1,000 μl) were mixed with one volume of absolute ethanol (500 μl) and added to a mini-spin column (Econospin; Epoch Life Sciences, Missouri City, TX, U.S.A.). The column was spun at 8,000 rpm for 1 min followed by two rinses with a washing buffer ("home-made AW2") (described as washing buffer I by Lemke et al. [2011]). After a 5-min drying period, 100 μl of elution buffer (10 mM Tris and 0.5 mM EDTA, pH 8) was added to the column, incubated at room temperature for 2 min, and then spun (6,000 rpm for 1 min) to obtain the final DNA extract. The DNA extract obtained in this manner is referred to as "NaOH purified" This process resulted in a DNA extract that was theoretically 20 \times more concentrated than that of the NaOH crude extracts.

Total concentration of extracted DNA was estimated for the CK and the NaOH crude and purified extracts using a fluorometer (Qubit 3; Thermo Fisher Scientific, Hampton, NH, U.S.A.) following the manufacturer's instructions. A subset was also analyzed by a microvolume spectrophotometer (NanoDrop 2000; Thermo Fisher Scientific, Waltham, MA, U.S.A.) for both DNA concentration and purity calculations (ratios of absorbance at 260 and 28 nm [A_{260}/A_{280}] and 260 and 230 nm [A_{260}/A_{230}]).

The limit of detection of *B. fagacearum* DNA present in extracted DNA samples from oak were determined using a dilution series. For the positive control, a series was made using pathogen DNA extract from a pure culture and obtained using the QIAmp Stool Kit, standardized for DNA concentration, and added in serial dilution to molecular-grade water. An equivalent dilution series was made by adding the pure pathogen DNA extract to a crude background solution (DNA extracted using the

NaOH crude protocol from sapwood shavings from healthy northern pin, bur, and white oak branches). The extracted pathogen DNA for water controls ($n = 4$) and for the pathogen DNA suspended in crude pathogen-free DNA ($n = 12$) were subjected to nested PCR amplification and gel visualization as described below.

Nested PCR and DNA sequencing. The DNA extracts obtained (CK, NaOH crude, and NaOH purified) were subjected to nested PCR as per the protocol described by Yang and Juzwik (2017), with two modifications. The PCR mixture was modified by adding 0.1% bovine serum albumen (BSA) and 1% polyvinyl pyrrolidone-40 (PVP) (Sigma-Aldrich, St. Louis, MO, U.S.A.), as described by Xin et al. (2003). These additions were used to increase efficiency of amplification in the PCR assays using crude DNA extracts obtained from pine needles and cotton leaves in a previously published report (Xin et al. 2003). In each full-plate reaction, PCR was performed with two negative (water) controls that lacked template DNA and one positive control with DNA extracted from known *B. fagacearum*.

For all experiments, products from the second round of PCR were visualized on 1.5% agarose gels with ethidium bromide staining. A nested PCR product was deemed positive if it produced a gel band of 280 bp (i.e., amplicon size). In general, the PCR assays were performed in two technical replicates (i.e., two PCR assays with the same batch of extracted DNA). A sample was deemed positive if either one or both samples produced the appropriate gel band. This approach has been used for other PCR-based diagnostic tests (Parra et al. 2020; Pilotti et al. 2012). Representatives of amplicon size-based "positive" samples were confirmed as pathogen positive via DNA sequencing using protocols described by Yang and Juzwik (2017). Obtained sequences were evaluated for quality and trimmed as appropriate. BLASTn searches in GenBank (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) were performed with the resultant sequences to identify *B. fagacearum* based on closely matched fungal accessions in the database (using 98% or higher identity for sequences greater than 200 bp).

Statistical analyses. A generalized mixed-effects model was used to identify differences in the probability of a *B. fagacearum*-positive PCR result between the three DNA extraction methods and between different oak species. Branch segments were treated as the experimental unit. The mixed-effects model was fit using DNA extraction method and oak species as fixed effects and with two random effects—one at the branch level and one at the tree level—to account for correlation between segments sampled from the same branch or tree (Bates et al. 2020; R Core Team 2019). Posthoc pairwise comparisons and Tukey's honestly significant difference (HSD) test were conducted using the emmeans packages in R to identify where detection rates differed between species (Lenth 2019). A two-way analysis of variance (ANOVA) of transformed data (\log_{10}) was conducted to examine the effect of extraction method (CK and NaOH purified only) and oak species on the concentration of extracted DNA (R Core Team 2019).

Results

Pathogen detection by standard isolation method. For actively wilting northern pin oak trees ($n = 4$), *B. fagacearum* was isolated from 90% of assayed branch segments ($n = 48$) using standard pathogen isolation techniques (Table 1). In comparison, 40% of branch segments ($n = 48$) from 12 branches of actively wilting bur oak trees ($n = 4$) and 54% of segments ($n = 48$) from 12 branches of wilting white oak ($n = 4$) yielded the fungus. Differences among oak species were significant based on glm model

analysis ($P = 0.0098$). When the proportion of positive segments is viewed graphically, the frequency of four segments per branch yielding the pathogen was high (75%) for northern pin compared with similar, low frequency (17%) of this occurrence in bur and white oak (Fig. 1A).

Pathogen detection by nested PCR using different DNA extraction methods. For the three extraction methods evaluated, resulting DNA was subjected to the same nested PCR protocol as described by Yang and Juzwik (2017), with the addition of BSA and PVP. For DNA extraction completed using the CK, 100% of the assayed northern pin oak branch segments ($n = 48$) were positive for the pathogen based on the presence of a 280-bp band on agarose gels produced from PCR products. In addition, all were positive on each of two technical PCR replicates. In comparison, the pathogen detection rates as determined by the CK were lower for bur oak (67%) and white oak (54%) compared with the rate for northern pin oak (Table 1). Nested PCR results using DNA extracted via the NaOH crude and NaOH purified protocols varied by oak species. A high pathogen detection rate (98%) was obtained for 48 branch segments from northern pin oak by either method, while lower rates were found for bur (58 and 79%) and white oak (71 and 62%) of 48 assayed samples per oak species by the crude or purified method, respectively (Table 1). A representative gel (Fig. 2) using nested PCR products of the three different bur oak extractions shows the typical scattered pattern of positive samples. No differences in the likelihood of a positive detection by PCR were found among the different DNA extraction methods based on model estimates ($P = 0.361$) (Table 2). However, the likelihood of such detection did differ by oak species; for example, for northern pin oak compared with bur oak ($P < 0.001$). Molecular assay-based detection rates for white and bur oak samples were similar and significantly lower than detection rates for northern pin oak based on P values resulting from posthoc means comparisons (Tukey's

HSD lower and upper confidence limits of -0.56 and 2.42 , respectively, for bur oak; -0.93 and 2.09 for white oak; and 2.54 and 7.62 for northern pin oak). Based on graphical presentation of branch segment results (four per branch), lower frequencies of positive segments per branch were found for bur and white oak compared with northern pin regardless of DNA extraction method used for the nested PCR assay (Fig. 1B to D).

Total DNA obtained. Total DNA obtained from the drill shavings using the CK, NaOH crude, and NaOH purified extraction methods were compared using fluorometric analysis. However, inconsistent results were obtained with the crude DNA extracts and are not included in this report. When the concentration data were analyzed by both species and extraction protocol (two-factor ANOVA), extractions using the CK yielded higher mean concentrations of total DNA than those from the NaOH purified protocol for all three oak species ($P < 0.0001$) (Table 3). The overall mean DNA concentration using the CK was 0.81 ng/ μ l (standard deviation [SD] = 0.511), while the mean concentration using NaOH purified was 0.16 ng/ μ l (SD = 0.109). Interactions between factors were significant. DNA concentrations were lower for white oak samples than those from bur and northern pin oak with the CK extractions (Fig. 3). Northern pin oak extractions obtained using the NaOH purified protocol yielded lower DNA concentrations than bur oak.

Concentrations were within the published sensitivity limits for the fluorimeter high-sensitivity assay (lower detection limit 0.01 ng/ μ l; Qubit 3) but were generally below the threshold for the microvolume spectrophotometer (lower detection limit 2 ng/ μ l; Nanodrop 2000). Therefore, any attempt to determine DNA purity with the later instrument from A_{260}/A_{280} or A_{260}/A_{230} ratios would be unreliable.

Calculating limit of detection. Because pathogen DNA concentration in the crude extract could not be determined by the instruments available, the limit of *B. fagacearum* DNA detection

TABLE 1
Detection of *Bretziella fagacearum* in branches from actively wilting crowns of northern pin, bur, and white oak trees using standard isolation and nested PCR amplification of extracted DNA obtained by using three different protocols^a

Oak species, detection approach	Method ^b	Branch segments ^c		Branch level ^d	
		Assayed	Positive	Assayed	Positive
Northern pin					
Isolation	–	48	43	12	12
PCR	Commercial kit	48	48	12	12
PCR	NaOH purified	48	47	12	12
PCR	NaOH crude	48	47	12	12
Bur					
Isolation	–	48	19	12	9
PCR	Commercial kit	48	32	12	11
PCR	NaOH purified	48	38	12	12
PCR	NaOH crude	48	28	12	12
White					
Isolation	–	48	26	12	10
PCR	Commercial kit	48	26	12	11
PCR	NaOH purified	48	30	12	9
PCR	NaOH crude	48	34	12	11

^a Data shown are total number branch segments assayed and number found to be positive, as numbers for when segment data were compiled by branch; – indicates not applicable.

^b DNA extraction methods. Commercial kit = QIAmp DNA Stool Kit, Qiagen; NaOH purified and NaOH crude extractions of DNA are based on modifications of protocol by Xin et al. (2003) and Lemke et al. (2011).

^c PCR positive based on results of two technical replicate PCR runs.

^d Results of branch segment assays were composited by branch.

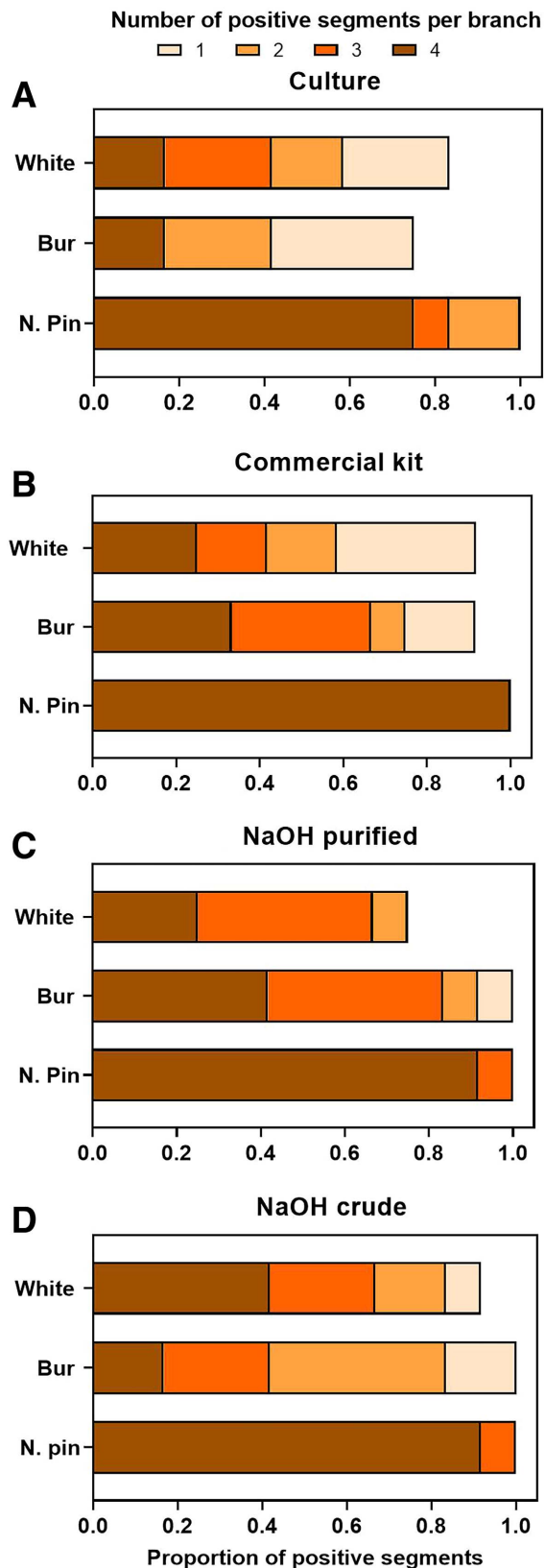


FIGURE 1

Detection frequencies for *Bretziella fagacearum* in branches of actively wilting northern pin, bur, and white oak. Numbers of positive branch segments per branch of each oak species are shown for **A**, a standard isolation assay, and for molecular PCR assays using three DNA extractions methods: **B**, commercial kit; **C**, purified NaOH protocol; and **D**, crude NaOH protocol.

was estimated by adding pathogen DNA (obtained from pure cultures using the CK method) in a dilution series utilizing crude pathogen-free extract as a background. The latter was obtained using the NaOH protocol with drill shavings obtained from oak-wilt-free northern pin, bur, or white oak branches. The lower limit (approximately 50% detection) found was pathogen DNA at 10^{-6} ng/ μ l, with no detection occurring at 10^{-7} ng/ μ l based on nested PCR amplification (12 replicates/concentration) and gel visualization. Similar data were obtained for all three species of wood extracts (Fig. 4).

Discussion

In general, substitution of either the crude NaOH or purified NaOH protocol for DNA extraction in the nested PCR procedure performed as well as or better than the CK DNA extraction method in detecting *B. fagacearum* (i.e., success rate) in actively wilting branches of a red oak species (northern pin oak) and two white oak group species (bur oak and white oak). This is comparable with results reported by others who have used a similar NaOH extraction technique. Osmundson et al. (2013) were successful in extracting and identifying DNA from lyophilized cultures and sporocarps, and for amplification of microsatellite loci. In fact, in several applications, the NaOH method performed as well or better than traditional cetyltrimethylammonium bromide methods.

Our detection results for northern pin oak samples were better than or similar to those reported by Yang and Juzwik (2017) for northern pin oak or northern red oak for nested PCR based on proportion of *B. fagacearum*-positive branch segments derived from total number of segments assayed from a branch. Using this same measure of detection frequency, our results were worse than those of Yang and Juzwik (2017) for CK use for DNA extraction for both bur and white oak in our study. These differences may be due to (i) the high variability of distribution of the fungus in the white and bur oak samples used in both studies or (ii) the skill and experience of the individual taking the drill shaving subsamples in the earlier study compared with the current one. For best results, drill shavings should be obtained from portions of the exposed xylem exhibiting characteristic vascular staining characteristic of *B. fagacearum* colonization.

The costs for performing the two NaOH extraction methods in this study were estimated to be \$0.62/sample for NaOH crude and \$1.60/sample for NaOH purified. This represents a reduction in cost when compared with that for the CK used (i.e., estimated to be \$6.47/sample). Additionally, time and cost savings of NaOH protocol use with single or small numbers of samples would not be as great as for high-throughput scenarios such as those developed by Lemke et al. (2011) and Xin et al. (2003). The NaOH procedure has been recommended by other researchers for barcoding, genotyping, and disease diagnostics (Osmundson et al. 2013) because of its speed, economy, and waste reduction. Waste reduction is reflected in the number of tubes needed (only one transfer is needed for the crude extract) and the lower amount of toxic wastes compared with traditional chloroform or phenol extractions. The potential also exists for our DNA extraction methods to be coupled with a PCR alternative procedure (e.g., gold nanoparticle enhanced chemiluminescence) (Singh et al. 2017), and the total amount of time to assay a small number of samples is reduced to less than 1 h in the laboratory.

One potential disadvantage of the NaOH crude extract is the presence of potential PCR inhibitors in the extract. The addition of BSA and PVP to the nested PCR mixture used in our studies did result in improved sharpness and brightness of bands on

agarose gels during visualization of the nested PCR products in all cases. Their use with DNA from the crude NaOH protocol made further purification (i.e., NaOH purified protocol) unnecessary in the case of northern pin and white oak. Xin et al. (2003) reported more efficient amplification of PCR mixtures in their work with crude DNA preparations from cotton leaves and from pine needles. Koonjul et al. (1999) suggested that BSA and PVP suppress certain substances in wood extracts (e.g., tannins and other polyphenols) that inhibit PCR assays. BSA but not PVP was used in the protocol evaluated by Osmundson et al. (2013) for multiple substrates.

Another potential disadvantage of the NaOH procedure is the low DNA concentration obtained. The amount of total DNA obtained using either NaOH protocol was less than that obtained using the CK; however, the NaOH-obtained DNA was sufficient to give generally comparable results in terms of success (i.e., detection of *B. fagacearum* DNA following PCR amplification). Crude extracts tended to have a light-brown or orange color, and assays performed by the fluorimeter were inconsistent and probably reflected contaminants and not true DNA concentrations. DNA concentrations below those detectable by the microvolume spectrophotometer hampered our ability to address any DNA

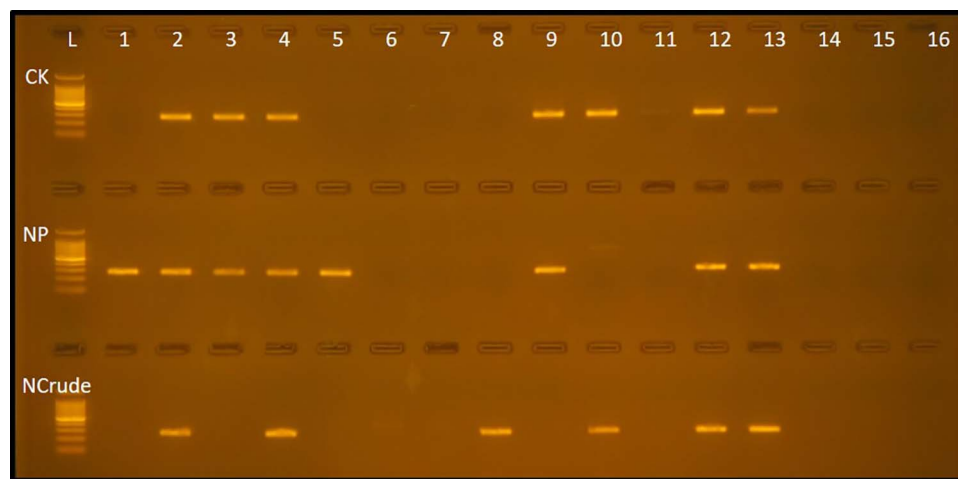


FIGURE 2

Agarose gel image showing results of nested PCR amplification of DNA extracted from bur oak using three different methods: CK = commercial kit, NP = NaOH purified, and NCrude = NaOH crude extract. Lane L: molecular size marker, 100 bp per line; lanes 1 to 12: DNA extracts from individual bur oak branch segments, each column from the same segment sample; lane 13: positive control, diluted *Bretziella fagacearum* DNA; lanes 14 and 15: negative control extract from known healthy bur oak wood; and lane 16: water control.

TABLE 2
Generalized linear mixed effects model of the interactions of actively wilting branches of northern pin, bur and white oak trees and DNA extraction methods used for nested PCR detection of *Bretziella fagacearum*^a

Variable	Level	Estimate	SE	Z value	P value
Intercept		0.8996	0.6545	1.375	0.1693
DNA extraction	NaOH purified	0.3255	0.3569	0.912	0.3618
	Commercial kit	-0.2243	0.3433	-0.653	0.5137
Oak species	Northern pin	4.1502	1.2261	3.385	0.0007
	White	-0.3569	0.8868	-0.402	0.6873

^a Extraction method NaOH crude and bur oak species are the reference levels. NaOH extraction were methods based on modification of protocols by Xin et al. (2003) and Lemke et al. (2011). Commercial kit = QIAmp DNA Stool Kit, Qiagen; SE = standard error.

TABLE 3
F test for fixed effects from analysis of variance of DNA concentration determined using two DNA extraction methods on sapwood drill shavings from *Bretziella fagacearum* infected northern pin, bur, and white oak trees^a

Fixed effects	df ^b	Mean square	F	Pr (> F)
Oak species	2	2.165	24.39	<0.0001
DNA extraction ^c	1	31.003	349.18	<0.0001
Species × DNA extraction	2	0.936	10.54	<0.0001

^a Analysis of variance was performed on log₁₀-transformed data in R (R Core Team 2019).

^b Degrees of freedom.

^c NaOH purified method based on Xin et al. (2003) and Lemke et al. (2011) was compared with commercial kit (QIAmp DNA Stool Kit; Qiagen).

quality and quantity questions. Nevertheless, the nested PCR technique allows one to start with a mixture containing minute quantities of target DNA, amplify the general fungal ITS DNA first, then amplify the specific target DNA for successful detection.

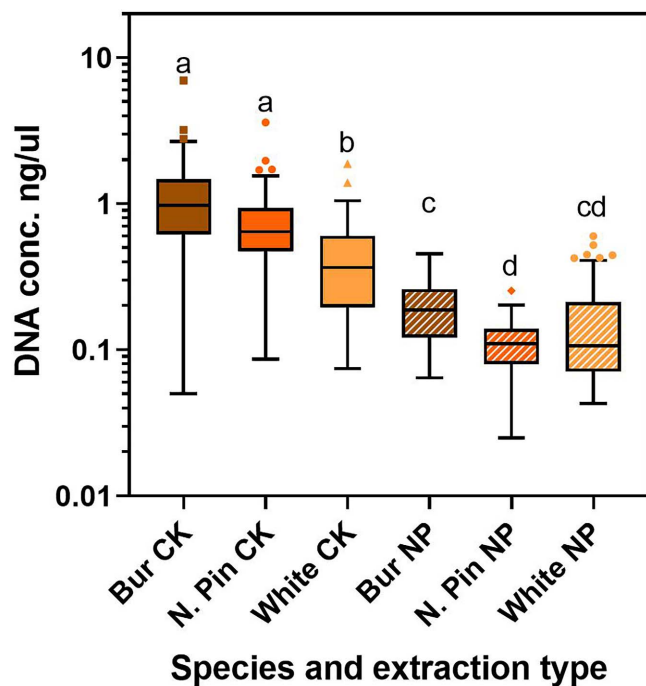


FIGURE 3

Box plots showing the concentration of total DNA obtained from *Bretziella fagacearum*-colonized sapwood of northern pin, bur, and white oak branches using two DNA extraction protocols: commercial kit (CK) or NaOH purified (NP). DNA concentration was measured using a fluorometer (Qubit 3). Different letters above plot columns indicate statistical difference at $P < 0.05$ based on analysis of variance and Tukey's method for means separation analyses of \log_{10} -transformed data.

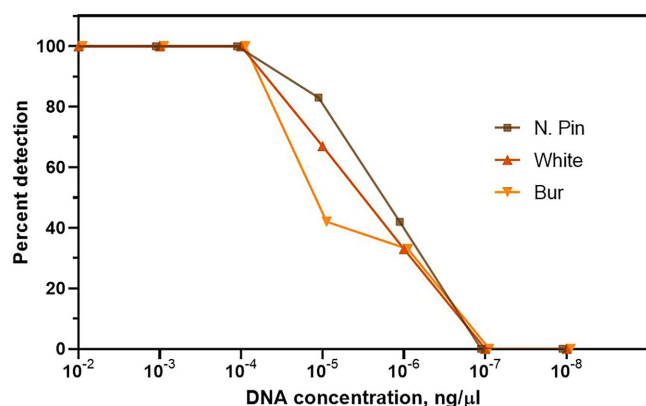


FIGURE 4

Limit of detection via nested PCR of *Bretziella fagacearum* DNA in NaOH crude extracts. Serial dilutions of pathogen DNA (extracted from pure culture using a commercial kit) were made in a matrix of NaOH crude extract of healthy northern pin, bur, and white oak wood samples. Percent detection is based on number of positive gel bands from 12 replicates per concentration level.

Statistically, the frequencies of pathogen detection using DNA extracted by the CK were no different than those obtained with NaOH purified DNA extracts for three oak species. Based on results of our limit of detection investigation, the lowest pathogen detection was found with extracts containing pathogen DNA at 10^{-6} ng/ul. Therefore, by inference, it is likely that the DNA present in most positive samples was 10^{-6} ng/ul or greater. We have occasionally encountered bur oak crude extract samples that required four technical replications of PCR runs in order to detect a positive sample (unpublished data). This suggests that *B. fagacearum* DNA concentration of those samples was probably at or below its lower level of detection. We hypothesize that the NaOH crude protocol performs best with oak sapwood samples with relatively high concentrations of the target *B. fagacearum* DNA concentrations.

Conclusions and Significance

In summary, substituting NaOH-based DNA extraction protocols for those in a CK resulted in similar detection rates for the oak wilt fungus in sapwood samples of three oak species when amplified using the same nested PCR protocols. Statistically, the crude extracts for all three species performed as well as the extracts that had been subjected to purification in spin filters. The clear advantages are reductions in time, expense, and waste. Potential disadvantage may be the limit of detection of the pathogen at very low concentrations, especially in the bur oak, or potential PCR inhibitors in the crude extract, either of which may be improved by the spin filter purification procedure and addition of BSA and PVP to the PCR. The alternative DNA extraction protocols may prove useful in future development of improved diagnostic methods for *B. fagacearum* detection in oak-wilt-suspect trees.

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