Use of sodium hydroxide DNA extraction methods for nested PCR detection of *Bretziella fagacearum* in the sapwood of oak species in Minnesota

Melanie J. Moore[1], Jennifer Juzwik[1], Olga Saiapina[2], Snober Ahmed[2], Anna Yang[1], and Abdennour Abbas[2]

[1]USDA Forest Service, Northern Research Station, St. Paul, MN 55108, U.S.A.

[2]Department of Bioproducts and Biosystems Engineering, University of Minnesota, St. Paul, MN 55108, U.S.A.

Corresponding author: J. Juzwik; E-mail: jennifer.juzwik@usda.gov

Funding: Minnesota Invasive Terrestrial Plants and Pests Center, University of Minnesota; USDA National Institute of Food and Agriculture Grant Number 00; Hatch project 1006789

ABSTRACT

Oak wilt caused by *Bretziella fagacearum* is an important disease of *Quercus* species, but 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. Polymerase chain reaction (PCR) tests can provide accurate lab diagnosis within two days. Two variations of a simple DNA extraction protocol using sodium hydroxide (NaOH) were compared to 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: DNA extraction, oak wilt fungus, *Quercus*

INTRODUCTION

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 oaks (*Quercus* species) 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 (NYS Department of Environmental Conservation 2020). Significant portions of Michigan, Minnesota, Texas, and Wisconsin have experienced on-going epidemics in recent decades, although disease suppression programs have also been underway in most of these states since the early 1990's (Juzwik et al. 2011). Oak wilt can dramatically alter both urban and natural ecosystems if left untreated (Appel 1995).

In red oaks (*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 site of pathogen introduction to the upper crown. Once infected, red oaks 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 oaks may not experience complete crown wilt for two to four years while infected white oaks may live for many years (Pokorny 2015).

Diagnosis of oak wilt is generally straight-forward 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 oaks 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 (*Agrilus 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 while affected white oaks 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. 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 to 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 1998, Yang and Juzwik 2017).

Currently, laboratory diagnosis of oak wilt is based on results from standard pathogen isolation (Pokorny 1999) or of 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). Commonly used molecular tests (arguably, the current "gold standard") involve extraction and purification of DNA followed by polymerase chain reaction (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 ITS1F/ITS4 and followed by further amplification with species-specific primers (CF01/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 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 commercial kit for DNA extraction is $6.47 USD per sample. Previously published methods involving use of a strong base, 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 NaOH extraction protocols of Wang et al. (1993) to work 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 neutralizing 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 amount of the target fungus present and/or rich in lignin or humic acids.

The purpose of this research was to evaluate the potential for substituting a NaOH-based DNA extraction protocol for the proprietary extraction protocol of a commercially available kit (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) to 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, Minnesota, in the Carlos Avery Wildlife Refuge, with actively wilting northern pin oak (*Q. 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 protocol of Yang and Juzwik (2017). Non-symptomatic branches from healthy northern pin oaks 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. Non-symptomatic branches from healthy bur and white oaks were sampled to use as controls. Segments (approximately 30 cm long) were obtained from each harvested branch, 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 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 presence of suspected colonies. Sub-cultures 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 oaks were processed within 7 days of collection, while samples from bur and white oaks were

processed within 12 days of branch harvest. Only those trees which produced *B. fagacrearum* positive cultures (four trees of each species) were used in the DNA extraction phase of the study.

At the same time 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 were placed in each of two 2 ml micro-centrifuge 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: 1) protocol of a commercial kit (QIAmp DNA Stool Kit, Qiagen, Venlo, The Netherlands), and 2) modified versions of published protocols using NaOH (Xin et al. 2003; Lemke et al., 2011).

For extractions using the kit, samples were processed as described in Yang and Juzwik (2017). In short, approximately 110 mg of drill shavings were placed in a 2 ml micro-centrifuge tube with two sterilized metal beads (4.5 mm in diameter). 750 ul Lysis buffer from the kit was added and the sample homogenized and then heated at 70^{o}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, eluting), except that that buffer quantities were reduced to 75% in order to match drill shaving sample sizes with the NaOH extraction.

For extraction of "crude" DNA using NaOH, approximately 110 mg of drill shavings were placed in a micro-centrifuge tube and 1.0 ml of 0.5 N NaOH (Buffer A described in Xin et al. 2003) added to sufficiently immerse the shavings (proportion used: 1 ml NaOH to 110 mg wood

168 shavings). Vigorous agitation with a vortex mixer was performed for 2 min, and then several
169 times for 2 to 3 seconds during 10 min of soaking in NaOH. The mixture was then centrifuged
170 (13,000 rpm for 2 min) and the resulting supernatant mixed with 100 mM Tris-HCl (pH 4.0)
171 (Buffer B as described in Xin et al., 2003) at a ratio of 1 to 9 (supernatant to buffer). The DNA
172 extract obtained in this manner is referred to as "NaOH Crude."

173 To obtain a purified and concentrated DNA extract, the NaOH crude extract was subjected to
174 a spin filter step that is similar to that used with the commercial kit. Specifically, two volumes of
175 the crude extract (1000 ul) were mixed with one volume absolute ethanol (500 ul) and added to a
176 mini-spin column (Econospin, Epoch Life Sciences, Missouri City, TX). The column was spun
177 at 8,000 rpm for 1 min followed by two rinses with a washing buffer ("home-made AW2")
178 (described as Washing Buffer I in Lemke et al. 2011). After a 5 min drying period, 100 µl of
179 elution buffer (10 mM Tris, 0.5 mM EDTA pH 8) was added to the column, incubated at room
180 temperature for 2 min, and then spun (6,000 rpm for 1 min) to obtain the final DNA extract. The
181 DNA extract obtained in this manner is referred to as "NaOH Purified." This process resulted in
182 a DNA extract that was theoretically 20 times more concentrated than that of the NaOH crude
183 extracts.

184 Total concentration of extracted DNA was estimated for the commercial kit and the NaOH
185 crude and purified extracts using a fluorometer (Qubit 3, Thermo Fisher Scientific, Hampton,
186 NH) following the manufacturer's instructions. A subset was also analyzed by a microvolume
187 spectrophotometer (NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA) for both DNA
188 concentration and purity calculations (A_{260}/A_{280} and A_{260}/A_{230} ratios).

189 The limit of detection of *B. fagacearum* DNA present in extracted DNA samples from oak
190 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 (commercial kit, NaOH crude, NaOH purified) were subjected to nested PCR as per the protocol described by Yang and Juzwik (2017) with two modifications. The PCR reaction mixture was modified by adding 0.1% bovine serum albumen (BSA) and 1% polyvinyl pyrroliodone-40 (PVP) (Sigma-Aldrich, St. Louis, MO) as described by Xin et al. (2003). These additions were used to increase efficiency of amplification in the PCR reactions 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 reactions were performed in two technical replicates, i.e. two PCR reactions 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 in Yang and Juzwik (2017). Obtained sequences were evaluated for quality and trimmed as appropriate. BLASTn searches in GenBank (www.blast.ncbi.nlm.nih.gov/) 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). Post-hoc pairwise comparisons and Tukey's HSD test were conducted using the emmeans packages in R to identify where detection rates differed between species (Lenth 2019). A two-way ANOVA of transformed data (log10) was conducted to examine the effect of extraction method (commercial kit 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 oaks (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 to 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 commercial kit, 100% of the assayed northern pin oak branch segments (n = 48) were positive for the pathogen based on 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 commercial kit were lower for bur oak (67%) and white oak (54%) compared to the rate for northern pin oak (Table 1). Nested PCR results using DNA extracted via the NaOH crude and NaOH purified protocol 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 (Figure 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, i.e. for northern pin oak compared to 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 post-hoc means comparisons (Tukey's HSD lower and upper CL of -0.56 and 2.42 and of -0.93

and 2.09 for bur and white oak, respectively; 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 to northern pin regardless of DNA extraction method used for the nested PCR assay (Fig. 1 B – D).

Total DNA obtained. Total DNA obtained from the drill shavings using the commercial kit and the NaOH crude and 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 commercial kit 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 commercial kit was 0.81 ng/ul (SD = 0.511), while the mean concentration using NaOH purified was 0.16 ng/ul (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 commercial kit 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 0.01ng/ul, Qubit 3), but were generally below the threshold for the microvolume spectrophotometer (lower detection limit 2 ng/ul, 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. Since pathogen DNA concentration in the crude extract could not be determined by the instruments available, the limit of *B. fagacearum* DNA detection was

284 estimated by adding pathogen DNA (obtained from pure cultures using commercial kit method)
285 in a dilution series utilizing crude pathogen-free extract as a background. The latter was obtained
286 using the NaOH protocol with drill shavings obtained from oak wilt-free northern pin, bur or
287 white oak branches. The lower limit (approximately 50% detection) found was 10^{-6} ng pathogen
288 DNA/ µl with no detection occurring at 10^{-7} ng/µl based on nested PCR amplification (twelve
289 replicates per concentration) and gel visualization. Similar data was obtained for all three species
290 of wood extracts (Figure 4).
291

292 **DISCUSSION**

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

302 Our detection results for northern pin oak samples were better than, or similar to, those
303 reported by Yang and Juzwik (2017) for northern pin oak or northern red oak for nested PCR
304 based on proportion of *B. fagacearum*-positive branch segments derived from total number of
305 segments assayed from a branch. Using this same measure of detection frequency, our results
306 were worse than those of Yang and Juzwik (2017) for commercial kit use for DNA extraction for

both bur and white oak in our study. These differences may be due to 1) the high variability of distribution of the fungus in the white and bur oak samples used in both studies, and/or 2) to the skill and experience of the individual taking the drill shaving sub-samples in the earlier study compared to 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 to that for the commercial kit 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 to traditional chloroform/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 one hour 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 reactions. 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 commercial kit; 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 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.

Statistically, the frequencies of pathogen detection using DNA extracted by the commercial kit 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 10^{-6} ng/ul pathogen DNA. 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 commercial kit 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 oaks, and/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 reaction. The alternative DNA extraction protocols may prove useful in future development of improved diagnostic methods for *B. fagacearum* detection in oak wilt-suspect trees.

ACKNOWLEDGEMENTS

We gratefully acknowledge the help of Melissa Mendez Carrejo and Theresa Garrison for lab and field support on this project, Paul Castillo for field support, and the cities of Eagan, Apple Valley, and Minneapolis for personnel and equipment. The authors thank Deborah Samac for manuscript review and access to and guidance in use of the microvolume spectrophotometer in her laboratory. The authors also acknowledge helpful comments of two anonymous reviewers.

The Institute for Research in Statistics at the University of Minnesota, and in particular Dr. Lan Liu, helped with the design and analysis of the experiments for this study.

LITERATURE CITED

Appel, D. N. 1995. The oak wilt enigma: Perspectives from the Texas epidemic. Annu. Rev. Phytopathol. 33:103-118.

Barnett, H. L. 1953. Isolation and identification of the oak wilt fungus. W. Va. Exp. Stn. Bull. 359T.

Bates, D., Mächler, M., Walker., S. 2020. Fitting linear mixed-effects models usint lme4. Journal of Statistical Software. 67(1):1-48.

Gould, A. B., and Lashomb, J. H. 2005. Bacterial leaf scorch of shade trees. APSnet Features, The American Phytopathological Society, St. Paul, MN. November 2005. https://apsnet.org/.

Haack, R. A., and Acciavatti, R. E. 1992. Twolined chestnut borer. USDA Forest Service Forest Insect and Disease Leaflet 168. USDA Forest Service, North Central Forest Experiment Station, East Lansing, MI.

Harrington, T. C., McNew, D., and Yun, H. Y. 2012. Bur oak blight, a new disease on *Quercus macrocarpa* caused by *Tubakia iowensi*s sp. nov. Mycologia 104:79-92.

Jensen-Tracy, S., Kenaly, S., Hudler, G., Harrington, T., and Logue, C. 2009. First report of the oak wilt fungus, *Ceratocystis fagacearum*, in New York State. Plant Dis. 93 :428.

Juzwik, J., and Appel, D. N. 2016. Oak wilt. Pages 129-133 in: Bergdahl, A. and Hill, A. tech. cords., Diseases of Trees in the Great Plains. General Technical Report RMRS-GTR-335, USDA Forest Service, Rocky Mountain Research Station, Fort Collins, CO.

Juzwik, J., Appel, D. N., MacDonald, W. L., and Burks, S. 2011. Challenges and successes in managing oak wilt in the United States. Plant Dis. 95:888-900.

Koonjul, P. K., Brandt, W. F., Lindsey, G. G., and Farrant, J. M. 1999. Inclusion of polyvinylpyrrolidone in the polymerase chain reaction reverses the inhibitory effects of polyphenolic contamination of RNA. Nucleic Acids Res. 27:915-916.

Lemke, L., Rex, M., Zyprian, E., and Töpfer, R. 2011. A simple, inexpensive and environmentally friendly method for high throughput DNA extraction from grapevine (*Vitis* spp.). Vitis 50:7-10.

Lenth, R. 2019. emmeans: estimated marginal means, aka least-square means. R package version 1.4.

New York State Department of Environmental Conservation 2020. Oak wilt. Division of Lands and Forests, Department of Environmental Conservation, Albany, NY. https://dec.ny.gov/lands/46919.html.

423 Osumndson, T.W., Eyre, C.A., Hayden, K.M., Dhillon, J., and Garbelotto, M.M. 2013. Back to
424 basics: an evaluation of NaOH and alternative rapid DNA extraction protocols for DNA
425 barcoding, genotyping and disease diagnostics from fungal and oomycete samples. Molec. Ecol.
426 Res. 13:66-74.
427
428 Parra, P. P., Dantes, W., Sandford, A., de la Torre, C., Pérez, J., Hadziabdic, D., Schaffer
429 and Gazis, R. 2020. Rapid Detection of the Laurel Wilt Pathogen in Sapwood of Lauraceae
430 Hosts. Plant Health Progress 21:356-364.
431
432 Pilotti, M., Lumia, V., Di Lernia, G., and Brunetti, A. 2012. Development of Real-Time PCR for
433 in wood-detection of Ceratocystis platani, the agent of canker stain of Platanus spp. European
434 Journal of Plant Pathology 134:61-79.
435
436 Pokorny, J. D. 2015. How to recognize common diseases of oaks in the Midwest: A quick guide.
437 NA-FR-01-15. USDA Forest Service, Northeastern Area State and Private Forestry, St. Paul,
438 MN.
439
440 R Core Team 2019. R: A language and environment for statistical computing. R Foundation for
441 Statistical Computing, Vienna, Austria. http://www.R-project.org.
442
443 Singh, R., Feltmeyer, A., Saiapina, O., Juzwik, J., Arenz, B., and Abbas, A. 2017. Rapid and
444 PCR-free DNA detection by nanoaggregation-enhanced chemiluminescence. Sci. Rep. 7(1)1-9.
445

446 Wang, H., Qi, M., and Cutler, A. J. 1993. A simple method of preparing plant samples for PCR.
447 Nucleic Acids Res. 21:4153.
448
449 Wu, C. P., Chen, G. Y., Li, B., Su, H., An, Y. L., Zhen, S. Z., and Ye, J. R. 2011. Rapid and
450 accurate detection of *Ceratocystis fagacearum* from stained wood and soil by nested and real-
451 time PCR. For. Pathol. 41:15-21.
452
453 Xin, Z., Velten, J. P., Oliver, M. J., and Burke, J. J. 2003. High-throughput DNA extraction
454 method suitable for PCR. BioTechniques 34:820-827.
455
456 Yang, A., and Juzwik, J. 2017. Use of nested and real-time PCR for the detection of *Ceratocystis*
457 *fagacearum* in the sapwood of diseased oak species in Minnesota. Plant Dis. 101:480-486.
458
459

460 **TABLES**

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. Data shown are total number branch segments assayed and number found to be positive, as numbers for when segment data were compiled by branch.

Oak species	Detection approach	DNA extraction Method[a]	Branch segments[b]		Branch level[c]	
			Assayed	Positive	Assayed	Positive
Northern pin	Isolation	--[d]	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/] 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.

[b/] PCR positive based on results of two technical replicate PCR runs.

[c/] Results of branch segment assays were composited by branch.

[d/] -- = not applicable

461

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* for data summarized in Table 1. Extraction method NaOH crude and bur oak species are the reference levels.

Variable	Level	Estimate	SE	Z-value	*P*-value
Intercept		0.8996	0.6545	1.375	0.1693
DNA extraction[a]	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/] NaOH extraction methods based on modification of protocols by Xin et al. (2003) and Lemke et al. (2011). Commercial kit = QIAmp DNA Stool Kit, Qiagen.

462 **Table 3.** F-test for fixed effects from analysis of variance of DNA concentration determined
463 using two DNA extraction methods on sapwood drill shavings from *Bretziella fagacearum*
464 infected northern pin, bur and white oak trees.[a]

Fixed effects	Df[c]	Mean Square	F	Pr(>F)
Oak species	2	2.165	24.39	< 0.0001
DNA extraction[b]	1	31.003	349.18	< 0.0001
Species*DNA extraction	2	0.936	10.54	< 0.0001

[a]/ ANOVA was performed on $\log_{10}$ transformed data in R (R Core Team 2019).

[b]/ NaOH purified method based on Xin et al. (2003) and Lemke et al. (2011) was compared with commercial kit (QIAmp DNA Stool Kit, Qiagen).

[c]/ Df = degrees of freedom

465

466

FIGURE CAPTIONS

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) 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.

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; NCrude: NaOH crude extract. Lane L: Molecular size marker, 100 bp per line; Lanes 1-12: DNA extracts from individual bur oak branch segments, each column from the same segment sample; Lane 13: Positive control, diluted *B. fagacearum* DNA; Lanes 14-15: Negative control extract from known healthy bur oak wood; Lane 16: Water control.

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 ANOVA and Tukey method for means separation analyses of $\log_{10}$-transformed data.

Figure 4. Limit of detection via nested PCR of *B. 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.

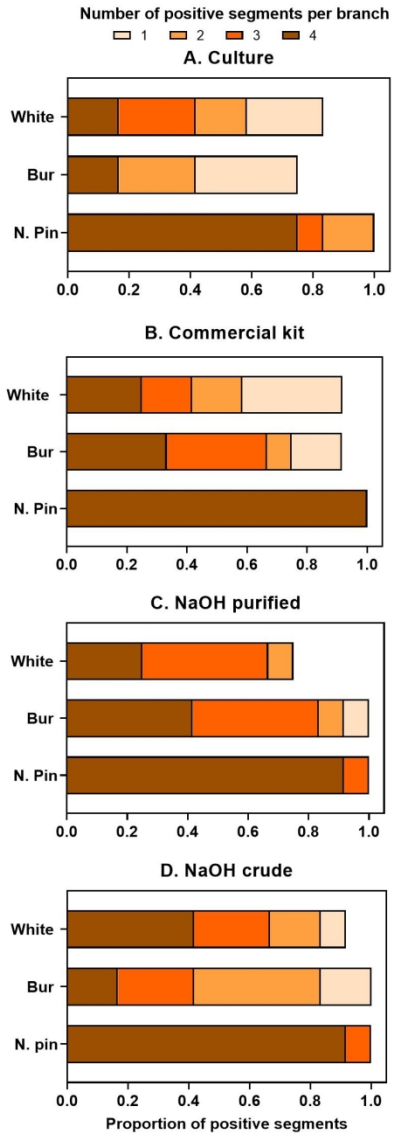

Figure 1. Detection frequencies for Bretziella fagacearum in branches of actively wilting northern pin, bur and white oak. Numbers of positive branch segments per branch of each oak species are shown for (A) standard isolation assay, and for molecular PCR assays using three DNA extractions methods: (B) commercial kit, (C) purified NaOH protocol, and (D) crude NaOH protocol.

82x201mm (300 x 300 DPI)

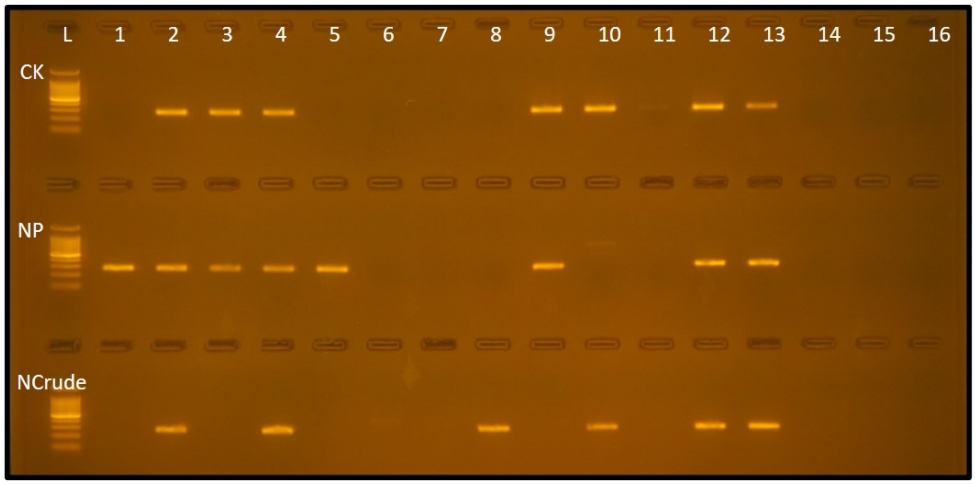

Figure 2. Agarose gel image showing results of nested PCR amplification of DNA extracted from bur oak using three different methods. CK: Commercial kit; NP: NaOH purified; NCrude: NaOH crude extract. Lane L: Molecular size marker, 100 bp per line; Lanes 1-12: DNA extracts from individual bur oak branch segments, each column from the same segment sample; Lane 13: Positive control, diluted B. fagacearum DNA; Lanes 14-15: Negative control extract from known healthy bur oak wood; Lane 16: Water control.

184x88mm (150 x 150 DPI)

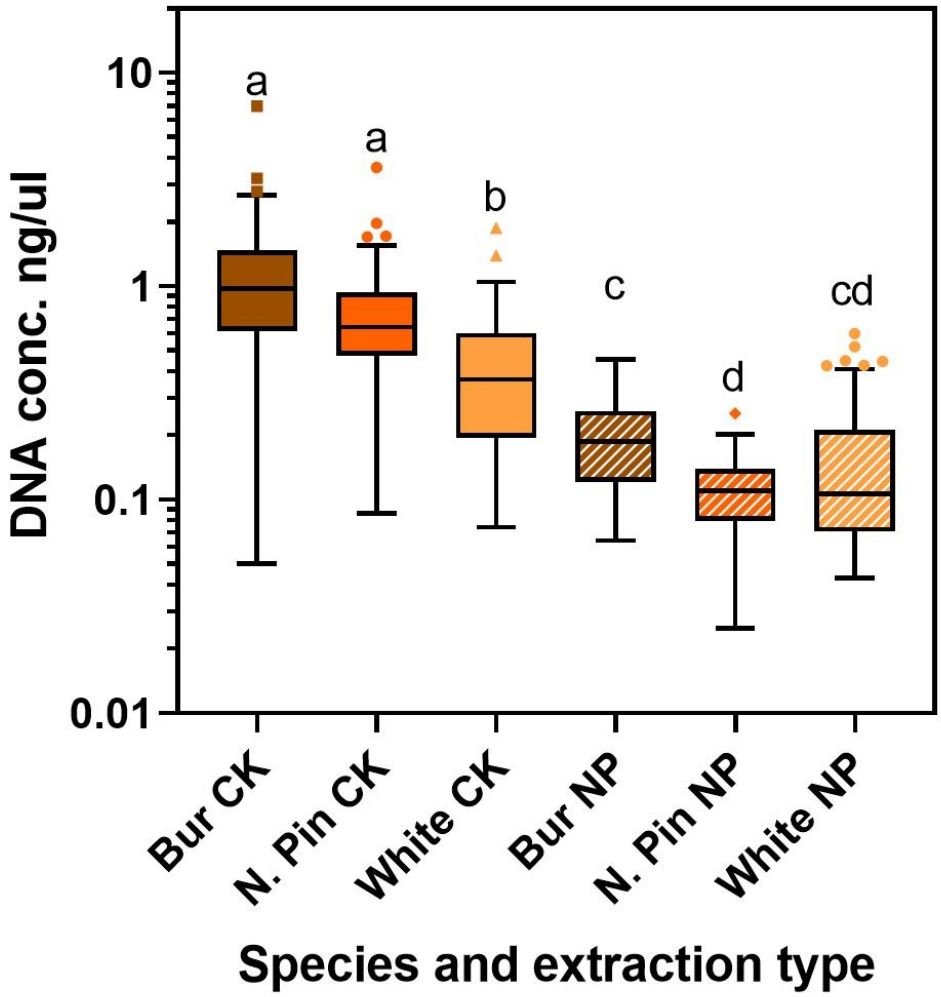

Figure 3. Box plots showing the concentration of total DNA obtained from Bretziella fagacearum colonized sapwood of northern pin, bur, and white oak branches using two DNA extraction protocols: commercial kit (CK) or NaOH purified (NP). DNA concentration was measured using a fluorometer (Qubit 3). Different letters above plot columns indicate statistical difference at $P<0.05$ based on ANOVA and Tukey method for means separation analyses of log10-transformed data.

82x90mm (300 x 300 DPI)

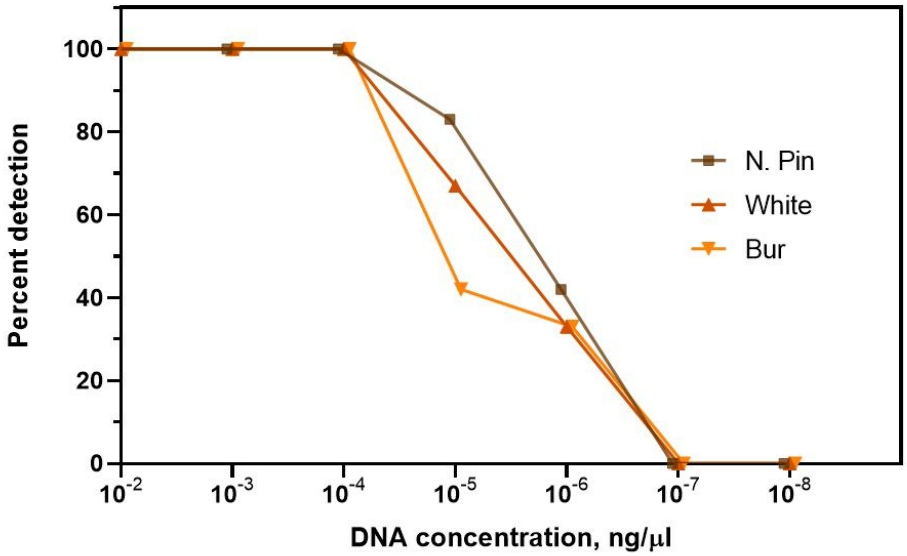

Figure 4. Limit of detection via nested PCR of B. fagacearum DNA in NaOH crude extracts. Serial dilutions of pathogen DNA (extracted from pure culture using a commercial kit) were made in a matrix of NaOH crude extract of healthy northern pin, bur and white oak wood samples. Percent detection is based on number of positive gel bands from 12 replicates per concentration level.

82x50mm (300 x 300 DPI)