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Fungal Basidiomycete *Ceratobasidium theobromae* DNA obtained directly from cocoa petioles

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Abstract. Junaid M, Purwantara A, Guest G. 2021. Fungal Basidiomycete *Ceratobasidium theobromae* DNA obtained directly from cocoa petioles. *Biodiversitas* 22: 2838-2843. Understanding the biology of the fastidious Basidiomycete *Ceratobasidium theobromae* occupying host-tissue is essential for plant disease management. Direct pathogen DNA extraction from infected plant tissue avoids the need for isolation in artificial media. We report a modified DNA isolation protocol to obtain total plant DNA designed to overcome DNA extraction and isolation problems caused by infected petioles rich in polysaccharides and phenolic substances as a primary source of gummosis. This study examined and compared total plant DNA isolated from petioles high in polysaccharides and polyphenolic compounds using two methods: conventional CTAB lysis buffer and Kits (the standard method), and a new modified CTAB protocol to address these PCR inhibitors. The modified method resulted in higher quality and quantity of *C. theobromae* crude DNA and amplified PCR product. The modified method produced large quantities of clear, transparent, aqueous DNA-containing lysate (crude DNA) with a clear separation between the upper crude DNA and organic waste layers. Mean DNA absorbance was 1.80, and the lowest DNA yield was 836.6 ng/ μ l. With the standard method, the blurred, viscous lysate obtained showed signs of gummosis, with poor separation between layers of crude DNA, polysaccharides, protein, and organic waste layers and low yield. Gel electrophoresis indicated poor quality DNA extract. We conclude that this modified method will be valuable for genetic diversity and disease studies in a range of previously challenging plant tissues and their pathogens.

Keywords: *Ceratobasidium theobromae*, cocoa petiole, modified plant-DNA-extraction, PCR inhibition

INTRODUCTION

Vascular streak dieback (VSD) is a disease affecting cocoa (*Theobroma cacao*) and is caused by Basidiomycete fungal pathogen *Ceratobasidium theobromae* (Guest and Keane 2007; Keane 1981; Samuels et al. 2012). Extracting total fungal (pathogenic) and plant DNA from infected cocoa tissues, such as leaves and stems, has a number of advantages. It avoids laborious preparations of cultures in media, reduces the risk of contamination, and avoids problems associated with purification. By amplifying fungal DNA regions directly from the total DNA extract of the infected plant tissue, the presence of the fungal pathogen in the plant is confirmed without the need for steps of isolation and purification of the fungus in nutrient media. However, successful plant DNA isolation is influenced by many factors, including methods, typical plant tissues, primary and secondary metabolites, and primary plant molecules (Castro-Moretti et al. 2020; Li, Y.M et al. 2013; Melnick et al. 2013; Piasecka et al. 2019; Porebski et al. 1997), while cross-contamination and cost-effectiveness are also (often related) issues (Deshui Yu et al. 2019).

As a natural consequence of plant genetics, biochemistry, or physiological responses to biotic and or abiotic interactions, the presence of metabolite compounds

is inevitable, along with derivatives as polysaccharides or polyphenolic substances (Agrios 2012; Castro-Moretti et al. 2020; Li et al. 2013; Melnick et al. 2013; Piasecka et al. 2019). In the cocoa plant, the petiole is the tissue producing the most polysaccharides and polyphenols once pathogenic infection occurs. On the one hand, pathogenic infection of leaves causes the host plant to release total polyphenolic compounds and other secondary metabolites at much higher rates (Santoso et al. 2017; Strobel et al. 2017). On the other hand, isolating pathogen DNA in the petiole tissue is an essential focus when tracing the pathogen within plant tissues, as it provides important information on the level of disease incidence and consequent loss of yield. The isolation of DNA from VSD fungal pathogens in cocoa stem tissue was previously reported (Junaid et al. 2020; Junaid and Guest 2021; Samuels et al. 2012), but the method used is less commonly applied to the petiole. The presence of VSD pathogen in the tissues is visible from dark staining on leaf scars after the twigs are initially infected, and the infection expands as the hyphae elongate through the vascular cambium (Guest and Keane 2007; Keane 1981; Marelli et al. 2019).

However, a primary issue in pathogen DNA isolation from petiole tissue is the high content of polysaccharides and phenolic compounds (Strobel et al. 2017). Therefore, attempts to achieve robust nucleic acid isolates are

challenging. This increase in plant chemical compound production is thought to be an active self-defence mechanism, resulting from phytoalexin and phytohormone activation; this mechanism can be triggered in several ways, including pathogen infection, pest infestation, or abiotic stress (Afifah et al. 2019; Castro-Moretti, Gentzel et al. 2020; Gómez et al. 2020; Lima et al. 2017; Pontes, Ohashi et al. 2016). These defensive compounds can become a significant inhibitory factor when seeking to extract high quality nucleic acids in sufficient quantity for genetic assays such as pathogen identification. Insufficient crude plant DNA yield at the initial isolation stage can cause a loss of amplicon quality during subsequent polymerase chain reaction (PCR) runs (Junaid 2018). This study tested the effectiveness of a modified plant DNA isolation protocol designed to obtain DNA of the fungal VCD pathogen *Ceratobasidium theobromae* directly from cocoa petioles through addressing the challenge posed by the presence of PCR inhibitors resulting from the disease.

MATERIALS AND METHODS

Plant DNA extraction and Isolation

In this study, all crude DNA extraction took place in Indonesia. The standard or conventional method used ready-made kit lysis buffers such as Isolate II plant DNA kit (#Bioline Cat: BIO-52070), CTAB (Henderson et al. 2013; Yu et al. 2019), RNase a 100mg biotech (Cat: BIORB0473-100mg) and proteinase K were used. The modified method of plant DNA extraction and isolation from infected cocoa petioles followed (Junaid 2018). The procedures used are described as follows:

Sample preparation

Petioles of interest (i. e. showing symptoms of VSD) were collected from cocoa orchards in three Indonesian provinces: South, West, and Southeast Sulawesi, from February to August 2017. The petioles (N= 100) were transported to the Lab of the Department of Plant Pest and Disease, Hasanuddin University, Makassar. To obtain sufficient heterogeneous and robust total DNA, the samples were placed in RNAlater® (Sigma-Aldrich) buffer as a stabilization solution to maintain tissue freshness during transport and storage (Junaid 2018). Each petiole was placed in a 2.0 mL cryovial containing 500 µL RNAlater solution. For short-term storage (up to 1 week), the petioles could be stored at room temperature while longer-term storage was kept between -5°C and -20°C. Each infected petiole was removed from its cryotube and blotted with a dry sterile paper towel in the laboratory, placed in a dry, sterile 2 mL cryovial. The tissue was freeze-dried under vacuum at -50°C for 48 h. If the sample was still moist, vacuum freeze-drying was continued for a further 72 h.

Pre-DNA isolation and extraction

Under the standard method, DNA from infected leaves was extracted and isolated using Bioline DNA kits and a modified CTAB lysis buffer. Under the modified protocol,

each vacuum-dried petiole was cut into small pieces and placed in a sterilized mortar with 75% dilute HCl before adding approximately 100 mg silica sand. The dried petiole was ground with a pestle to a fine powder with a floury texture (Figure 1). The product was transferred to a 2 mL microcentrifuge tube to which 700 µL preheated lysis CTAB buffer was added together with 30-50 µg lipolysis proteinase K powder.

The lysate was vortexed for 60-90 seconds to homogenize the sample and lysate buffer solution and then placed in a 65°C water bath for 100 to 150 min. In the original CTAB protocol (Doyle 1987), the incubation time needed was relatively short (about an hour), probably related to the type of specimen tested which did not include cocoa petioles. Therefore, this method was modified to enable DNA extraction from petioles. Increasing lysis incubation time at 65°C was found to be the primary key to obtaining high-quality lysate for further work, while insufficient lysis buffer led to poor results. During the water bath, the tube was inverted every 10 minutes and 200 µL sodium acetate was added before the second inversion. The lysis buffer preparation should retain a volume of around 2 mL, more than the sample size expected for extraction. If lysis buffer evaporated during the water bath, a further 200-300 µL of fixed lysis CTAB buffer was added to compensate for the loss of a significant volume of lysis buffer as the solution evaporated. A transparent aqueous lysate indicated a successful extraction.

Once a visible layer separating the lysate and waste material had formed, the lysate was pipetted carefully into clean microcentrifuge tubes, avoiding contamination of the sample with waste material. A chloroform and isoamyl alcohol (Chloroform) v/v mix (24:1) was added to the lysate and vortexed for 30-60 seconds until the lysate solution was thoroughly mixed, resulting in a milky white to light yellow emulsion. The lysate solution was centrifuged for 15 min at 12,000–14,000 rpm (room temperature). The aqueous layer (supernatant) was carefully pipetted into a sterilized 2 mL tube. Once prominent visible separate layers were seen (organic fraction at the bottom, interphase in the middle and uppermost aqueous transparent fraction), the next stage was to collect the layer containing the nucleic acids (DNA). A pipette tip was modified by snipping the end off to enlarge the opening and used to pipette the nucleic acid layer (supernatant) into a tube, carefully avoiding uptake of the organic and interphase layers. Chilled isopropanol (400 µL) and then 200 µL of sodium acetate 3 M (NaOAc) were added to the tube which was inverted for 10-20 seconds and incubated overnight at 29°C to induce nucleic acid precipitation. The sample was then centrifuged at 13,000–14,000 rpm (room temperature) to separate the nucleic acids, deposited as a crude DNA pellet, from the liquid waste. The liquid was carefully decanted and disposed of, then the tube with the DNA pellet adhering to the inner surface was inverted and placed on a sterilized tissue towel over a 60°C heating block for 5 minutes to ensure no residual liquid remained.

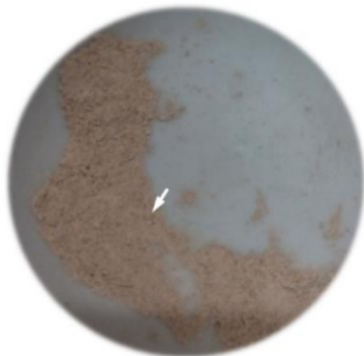


Figure 1. Smoothly ground tissue (white arrow) obtained by grinding petioles with a pestle and used in the modified plant DNA isolation protocol

The DNA pellet was cleaned by first adding 400 μL of 1% chilled ethanol and centrifuging for 60 seconds at 14,000 rpm. The liquid was then gently decanted to avoid detachment of the precipitate (DNA pellet) from the tube which was then inverted for 10 minutes on a sterilized tissue towel. This was followed by a second rinse with 300 μL 96% EtOH and centrifuging for 3 minutes at 14,000 rpm. The retained DNA pellet was visible at the bottom of the vial and dried by inverting the tube over a heating block (65°C) for 5-10 min or leaving it at room temperature for 1-2 h.

DNA template solution was produced by adding 50 μL TE buffer as both a solvent and preservative, followed by rapid centrifuging for 15 seconds at 13,000 rpm to ensure all the nucleic acid was in solution. The presence and quality of nucleic acid present in the extracted DNA template were evaluated electrophoresis. The template solution was stored at -20°C.

PCR amplification

PCR amplification and analyses of DNA template and PCR product (amplicon) quality and quantity measurement were conducted in the Biomedical Building Lab, School of Life and Environmental Science, Faculty of Science, The University of Sydney. The DNA obtained from infected petioles was quantified using Nanodrop spectrophotometry (NanoDrop™ 2000/2000c Spectrophotometers) following UV/VIS protocol. This quantification was used to determine the amount of DNA template to be used in PCR amplification of a diagnostic molecular marker for *Ceratobasidium theobromae* using the specific primer pair Than_ITS1

(forward: 5'-TGGTCATTTAGAGGAAGTAA-3') and Than_ITS2 (reverse: 5'-CAGACTT(G/A)TA(C/T)ATGGTCCAG-3') (White et al. 1990 in Samuels et al. 2012).

A PCR master mix was prepared with 7.6 μL dH₂O, 7 μL 5 MyTaq Red reaction buffer, 2 μL primer forward (ITS1 10mM), 2 μL primer Reverse (ITS4 10 mM), and 0.4 μL MyTaq Red DNA polymerase (Bioline#Bio-2118). In

each PCR reaction tube, 1.5 μL of PCR master mix was combined with 0.1 μL sample DNA template. A reaction tube containing 2 μL PCR master mix without DNA template was used as a negative control, and for positive control, a 1.5 μL PCR mixture with 0.5 μL DNA template from a reference sample. The thermal cycler PCR profile comprised initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C (30 seconds), annealing at 55°C (1 minute) and extension at 72°C (1 minute), and a final extension at 72°C (10 min) following Samuels et al. (2012).

RESULTS AND DISCUSSION

The modified plant DNA isolation method produced DNA template of much higher quality than the standard isolation method (Figure 2). The quantity (yield) and quality of fungal Basidiomycete *Ceratobasidium theobromae* DNA differed between methods (Table 1).

Absorbance is a good indicator of DNA template quality and can be used to verify whether the DNA extracted is sufficient in terms of both quantity (> 100 ng/ μL) and quality ($\lambda = 1.8-2.0$) for proceeding to PCR amplification (Figure 3). The brightness and thickness of DNA fragments visualized through gel electrophoresis are a good indication of PCR product quality (Figure 4).

With regards to the standard protocol, the lysate exhibited gummosis, with no clear separation between the upper crude DNA layer and lower organic and plant debris layers, yielding relatively low quantities of turbid, viscous supernatant, as can be seen in Figure 2.B. The impurities remaining in the crude DNA extract were clearly visible in the “dirty” smeared appearance of the DNA template electrophoresis bands (Figure 3) which contrasted with the clear, bright bands of DNA template extracted using the modified method. These impurities, combined with an insufficient quantity of DNA (Table 1), were the central issues when using the standard method. Very few samples exhibiting the smeared electrophoresis bands typical of high levels of impurities such as those in Figure 3 were successfully amplified through PCR.

The modified method for extracting DNA from cocoa petioles appeared to avoid gummosis and other contamination problems, proving suitable to extract robust DNA templates from the samples of interest. The lysate produced exhibited three distinct, well-separated layers: aqueous fraction (supernatant), interphase and organic matter (Figure 2.A). Nucleic acids are water-soluble molecules and accumulate in the aqueous fraction; proteins and lipids accumulate in the organic fraction, and other plant materials are trapped in the interphase. In this modified technique, the aqueous fraction of the lysate was clear and transparent, comprising a high proportion of the centrifuged lysate and generally containing DNA in quantities suitable for PCR. The sharp bright bands typically observed from sample DNA template electrophoresis (agarose gel stained with ethidium bromide) reflect the quality of the DNA extracted.

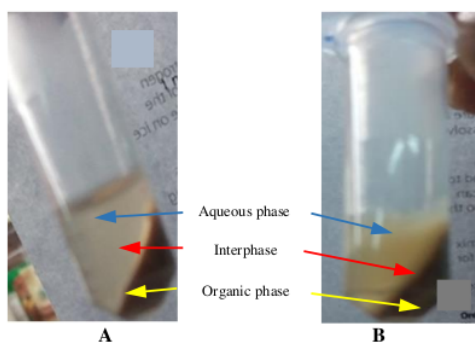


Figure 2. Nucleic acids (crude DNA/extracted DNA product) obtained from modified (A) and standard (B) DNA isolation methods. In the modified method (A), the supernatant was a transparent non-viscous fluid containing crude DNA (blue arrow), well separated from polysaccharides and other proteins in the intermediate layer (red arrow) and organic waste (yellow arrow) in the bottom layer. The standard DNA isolation method (B) produced lysate with gummosis and poor separation between the crude DNA layer (blue arrow) and the organic/plant debris layers (red and yellow arrows). The supernatant was turbid with higher viscosity and low amounts of crude DNA leading to PCR inhibition issues.

Spectrophotometric analysis is a frequently used method for testing DNA purity, with the $A_{260/280}$ UV absorbance ratio as the primary key. The UV/VIS absorbancy ratio deemed acceptable for DNA/RNA template purity ranges from 1.8-2.0 (Ali et al. 2019; Arruda et al. 2017; Dash 2013). In this study, measurements taken for selected samples with a Nanodrop spectrophotometer indicate that the DNA template extracted for fungal pathogen analysis of cocoa petiole tissue using the modified method had UV/VIS absorbance ratios in the 1.8-2.0 range, and therefore satisfied purity standards. The spectrophotographic absorbance data also quantified the DNA template with values in excess of 100 ng/ μ l. Data for selected samples of DNA template tested with spectrophotometry (Table 1) indicate that the modified method consistently produced DNA template of sufficient quality and quantity for PCR analysis. Conversely, all with the standard isolation method, the absorbancy ratio was less than 1 and the quantification values of 10 ng/ μ l or less indicate DNA template of insufficient quality and quantity for successful PCR amplification, e.g. to detect the fungal VCD pathogen *C. theobromae*.

Table 1. Quality and quantity of amplicons of selected samples obtained from two plant DNA isolation protocols

Sample	Region	District	Village	DNA quantity & quality	
				(ng/ μ l)	(λ 260/280)
Modified plant DNA isolation protocol					
993	West Sulawesi	Mamuju	Bebanga	2599.50	1.83
1837	South Sulawesi	Zharrang	Pakkie	1264.60	1.90
1122	West Sulawesi	Mamuju	Salokayu	1155.10	1.97
1095	West Sulawesi	Central Mamuju	Polopangale	1061.70	1.85
1058	West Sulawesi	Mamuju	Bebanga	1033.50	1.85
1121	West Sulawesi	Central Mamuju	Polopangale	1027.60	1.72
1555	West Sulawesi	Polman	Anreapi	998.10	1.99
1287	West Sulawesi	Central Mamuju	Kombiling	975.50	1.71
1111	West Sulawesi	Mamuju	Salokayu	900.30	1.94
1288	West Sulawesi	Central Mamuju	Budong-Budong	886.20	1.92
1648	South Sulawesi	Barru	Tompo	881.50	1.90
1832	South Sulawesi	Barru	Pattappa	871.90	1.78
1107	West Sulawesi	Central Mamuju	Polopangale	861.90	1.92
1195	West Sulawesi	Central Mamuju	Polopangale	837.70	1.90
967	West Sulawesi	Central Mamuju	Salumanurung	836.60	1.94
Standard plant DNA isolation protocol					
58	South Sulawesi	East Luwu	Laro	1.00	-3.75
63	South Sulawesi	East Luwu	Lembara Harapan	55.30	0.97
76	Southeast Sulawesi	North Kolaka	North Kolaka	0.50	-1.53
85	Southeast Sulawesi	North Kolaka	North Kolaka	1.40	-3.04
90	Southeast Sulawesi	North Kolaka	North Kolaka	1.30	-68.48
94	Southeast Sulawesi	North Kolaka	North Kolaka	0.50	0.67
98	Southeast Sulawesi	North Kolaka	North Kolaka	3.00	1.00
184	Southeast Sulawesi	Kolaka	Kolaka	34.40	1.23
193	Southeast Sulawesi	North Kolaka	North Kolaka	0.60	-1.77
194	Southeast Sulawesi	North Kolaka	North Kolaka	1.40	-6.06
197	Southeast Sulawesi	North Kolaka	North Kolaka	-0.30	0.19
198	Southeast Sulawesi	North Kolaka	North Kolaka	-0.10	0.05
198	Southeast Sulawesi	North Kolaka	North Kolaka	0.40	-1.21

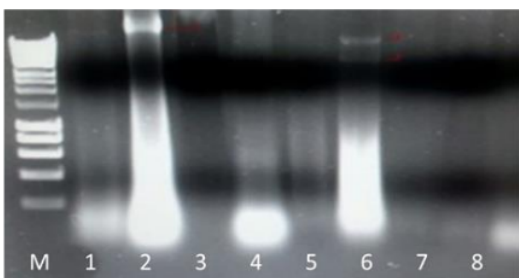


Figure 3. Agarose gel electrophoresis stained with ethidium bromide demonstrating the poor quality of crude DNA obtained using the standard plant DNA isolation method. This DNA template was not successfully amplified with the specific primer pair Than_ITS1 and Than_ITS2; confirm⁶ on the presence of VSD pathogen was poor. M denotes the 100 bp marker; lane 1: negative control; lanes 2-8: sample DNA template.

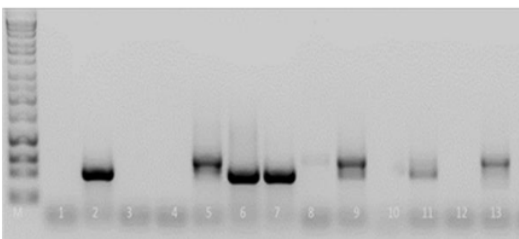


Figure 4. Agarose gel electrophoresis of PCR product obtained using the modified plant DNA isolation protocol and specific primer pair Than_ITS1 and Than_ITS4, and stained with ethidium bromide; the bands confirm⁶ the presence of the VSD disease pathogen. M denotes the 100 bp marker ladder; lane 1: negative control; lanes 2-13: sample DNA template.

The majority of DNA samples extracted using the modified protocol were consistently amplified by the specific primer PCR (Table 1; Figure 4). Some samples were not amplified, but this does not necessarily mean that plant and/or pathogen DNA was not present. One possibility is that the pathogen present was not *C. theobromae*, and therefore was not recognized and amplified by the primer pair used. It is also possible that there may be problems with the PCR or other processes, including the modified extraction process, for these specimens. However, the detection rate was greatly improved using this modified protocol (Figure 4).

Seeking effective DNA isolation techniques to overcome gummosis due to increased polysaccharide and phenolic compounds in the cocoa petiole is an emerging challenge. Solutions proposed include the addition of sodium chloride and potassium acetate before transfer to ice-cold absolute isopropanol for DNA precipitation to reduce polysaccharides (Aboul-Ftooh et al. 2019). However, while this protocol seems effective in soybean and corn, it is not effective for cocoa petioles. In our modified protocol, factors contributing to a reduction in the

risk of gummosis due to the increase in polysaccharides and other substances include the immediate use of the RNAlater buffer (SigmaAldrich product code R0901) when collecting samples in the field (before loading samples into the freeze drier) and increasing the volume of CTAB buffer during lysis and precipitation (Figure 2).

One reason behind the success of this modified protocol is that aqueous RNAlater not merely inactivates the sources of enzymes in the petiole responsible for the rise of polysaccharide and phenolic compounds but can also maintain tissue freshness, thereby greatly improving DNA yield and quality. The infected petiole is simply transferred into a 2 mL vial and submerged in RNAlater. This is a significant improvement over the use of standard buffers such as ethanol or distilled water, which can allow or promote rapid tissue gummosis. Furthermore, the longer the infected petiole is kept in the vial, the more gummosis occurs, worsening the inhibition of DNA isolation (Figure 2B) and PCR amplification. In contrast, using the modified protocol, RNAlater can preserve tissue ⁵hour gummosis affecting DNA extraction for up to 1 day at 37°C, one week at 25°C, and one month at 4°C. Tissues can also be stored long-term at -20°C.

The lysis buffer is also an important factor, in terms of formulation and volume. For example, the addition of 2-β-mercaptoethanol and polyvinylpyrrolidone has been found to reduce the production of secondary metabolites from plant tissues and can significantly improve DNA purity (Aboul-Ftooh et al. 2019; Arruda et al. 2017), while increasing lysis buffer volume can lead to improved DNA quality (Aboul-Ftooh et al. 2019; Henderson et al. 2013). The increased lysis buffer volume (200-300 μL) in the initial stage with extra incubation time (40-90 min), the higher concentration (90-95%) of chilled isopropanol or ethanol and pipette tip modification (a blunt tip cut) are other modifications contributing to the robust production of DNA samples using the modified method. Gummosis is still a risk in every single preparation, making the proteinase K and a sufficient extra volume of heated lysis buffer especially vital for achieving good results in the next stage. We found that it was better to use more lysis buffer (up to 1 mL) and if necessary, prepare an additional new vial when the crude DNA is transferred by pipette. Incubation up to 70°C for 60 min in a specific treatment vial can help the lysis buffer work more effectively, and if there are still signs of gummosis in the incubated lysate, returning the lysate for further incubation after adding more lysis CTAB buffer can enable the recovery of good quality DNA. Overnight incubation of the lysate before the crude DNA is washed with chilled isopropanol can also increase DNA quality.

In conclusion, the modified protocol enabled robust extraction of quality DNA templates (fungal pathogen and plant DNA) from cocoa petiole tissue with symptomatic VCD lesions. The aqueous fraction of the lysate containing crude DNA was clear, transparent, produced in large amounts, and well separated from the organic waste layers. This contrasted with the standard protocol lysate which exhibited signs of gummosis, with a turbid, viscous

aqueous fraction poorly separated from the organic waste layers and containing a low quantity of crude DNA. Agarose gel electrophoresis indicated and spectrophotometry confirmed the differences in crude DNA quality and yield. While gummosis was an issue using standard DNA extraction methods, the modified protocol produced clean DNA template which enabled a robust identification of the fungal pathogen *Ceratobasidium theobromae* through PCR using a specific primer pair. This modified method will be valuable for studies of genetic diversity in plants, especially challenging plant tissues such as petioles and associated microorganisms including pathogens.

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