Appendix 2C: DNA Extraction Optimisation

A range of DNA extraction procedures was assessed for the ability to efficiently and consistently extract both fungal and plant DNA from diseased tissues. DNA quality and its performance in the qPCR assay were also monitored. The procedures assessed were the CTAB method and Wizard and Qiagen DNA extraction kits.

Methods

Lyophilisation

Before extraction all tissues were lyophilised. Harvested plant tissue samples were chilled in a -70°C freezer for at least one hour. Samples were placed in a Virtis benchtop SLC lyophiliser for 48 hours at -40°C and less than 170mtorr. The vacuum was provided by a JAVAC JL-5 Laboratory series high vacuum pump. After lyophilisation the samples were re-weighed to allow dry weights to be determined. Samples were then ground to an homogenous powder using a TissueLyser (Qiagen, Doncaster, Victoria) and three tungsten carbide beads per tube. Sub-samples of large samples were placed into a tube new for DNA extractions. Each sample and sub-sample weight was recorded.

DNA Extraction

CTAB (cetyl trimethylammonium bromide) method

Cellular DNA was isolated by a modification of the method of Murray and Thompson (1980). Briefly, CTAB-buffer was added to a microfuge tube containing ground tissue to lyse the nuclear membrane. After incubation an aqueous phase was extracted using chloroform:isoamylalcohol (24:1 (v/v)) to remove proteins and cell debris. DNA was precipitated out of this aqueous phase by addition of isopropanol and centrifugation. The supernatant was discarded and the DNA pellet was washed with ethanol. The DNA pellet was rehydrated by addition of MilliQ water and overnight incubation. The DNA suspension was then treated with RNase, followed by precipitation using ammonium acetate and chloroform:isoamyl alcohol. After centrifugation the aqueous phase was removed and placed in 100% ethanol for overnight precipitation. After further centrifugation the DNA pellet was washed twice with 70% ethanol. The DNA was resuspended in MilliQ water.

Full CTAB DNA Isolation Method

DAY 1:

1. Freeze-dry young harvested leaves (or applicable material) and grind to fine powder.
2. 2x CTAB Buffer:

	Stock	**mL**	**mL**	**mL**	**mL**	**mL**	**mL**
ddH_2O	-	1.89	3.78	7.56	9.45	11.34	15.12
100mM Tris pH 8.0	1.0 M	0.5	1.0	2.0	2.5	3.0	4.0
20mM EDTA	0.5 M	0.2	0.4	0.8	1.0	1.2	1.6
1.4mM NaCl	5.0 M	1.4	2.4	5.6	7.0	8.4	11.2
2% CTAB	10.0%	1.0	2.0	4.0	5.0	6.0	8.0
0.2% ß-mercapto-ethanol	-	10 µL	20 µL	40 µL	50 µL	60 µL	80 µL
Total		**5mL**	**10mL**	**20mL**	**25mL**	**30mL**	**40mL**

3. Add 750µL CTAB-buffer to approximately 250µL of fine leaf powder in a 2mL microfuge tube.
4. Incubate at 65°C for one hour
5. Extract suspension with 500µL chloroform:isoamylalcohol (24:1 (v/v)). Mix well. Centrifuge at 12000g for 5 minutes.
6. Precipitate DNA from aqueous phase with 500µL (0.66 volumes) isopropanol. Mix well.
7. Incubate at room temperature for 20 min.
8. Centrifuge at 12000g for 5 min. Discard supernatant and drain tubes upside down.
9. Wash precipitate at room temperature by adding 500µL ice-cold 70% (v/v) ethanol. Incubate for 20min.
10. Centrifuge at 12000g for 5 min. Discard supernatant.
11. Air-dry pellet for one hour at room temperature.
12. Resuspend in 200µL TE buffer, pH 8.0 at room temperature for 1 hour or overnight at 4°C.

DAY 2:

13. Add 2 µL RNaseA (10mg/mL). Incubate at 37°C, 1-2 hours.
14. Precipitate DNA with 20µL 7.5 M ammonium acetate and an equal volume (200µL) chloroform:isoamylalcohol (24:1 (v/v). Mix well.

15. Centrifuge at 12000g for 5 min.
16. Precipitate DNA from the aqueous phase overnight with 500µL ice-cold 100% ethanol.

DAY 3:

17. Centrifuge 15 min at 12000g. Discard supernatant.
18. Wash 2x with ice-cold 70% ethanol (500µL) by centrifuging for 10min each time.
19. Discard supernatant, drain tubes upside down.
20. Air-dry pellet
21. Resuspend pellet in 50µL TE Buffer pH 8.0
22. Incubate at 37°C for 2 hours or overnight at 4°C.
23. Determine concentration and purity.
24. Run Agarose gel and dilute

Wizard

Wizard Genomic DNA Extraction kit (Promega, Sydney, New South Wales, Australia) was used to extract the fungal/plant DNA, following the plant DNA extraction protocol. Briefly, Nuclei Lysis solution was added to the ground tissue and vortexed to solubilise the nuclear membrane, releasing the DNA. After incubation RNase was added to the lysate to degrade any RNA. After further incubation, Protein Precipitation Solution was added. Centrifugation produced a pellet of precipitated proteins, with the supernatant containing the DNA. This supernatant was removed and added to a tube containing 100% isopropanol allowing the DNA to be isolated by centrifugation. The supernatant was removed and the DNA pellet was washed with ethanol. The DNA pellet was rehydrated by addition of DNA Rehydration Solution (autoclaved MilliQ water) and incubation.

Full Wizard DNA Extraction Method

Modified from the Wizard Genomic DNA Extraction for Plant Material Protocol

1. Add 600µL of Nuclei Lysis Solution
2. Vortex tube until an homogenous suspension is produced
3. Incubate tube for 25 minutes at 65°C
4. Add 12µL of RNase Solution (1ng/mL) and invert tube to mix contents
5. Heat contents for 15 minutes at 37°C
6. Remove tube from heat and allow to cool for 5 minutes before proceeding

7. Add 200µL of Protein Precipitation Solution to the tube and vortex for approximately 20 seconds
8. Centrifuge tube for 10 minutes at 14000rpm
9. Remove supernatant into a new tube and repeat step 8
10. Remove supernatant into another 1.5mL microfuge tube containing 600µL of 100% isopropanol
11. **Gently** invert tubes to precipitate DNA
12. Centrifuge for 2 minutes at 14000rpm (hinges in)
13. Remove supernatant and add 600µL of 70% Ethanol, gently invert tubes
14. Centrifuge for 2 minutes at 14000rpm (hinges out)
15. Remove supernatant and leave tube to sit open, upside down, for 30 minutes (until dry)
16. Add 200µL of DNA Rehydration Solution (autoclaved MilliQ water) and incubate tube at 65°C for 1 hour
17. Centrifuge tube for 5 seconds at low/medium speed before using sample for DNA quantification.

Qiagen

The procedure recommended in the Qiagen DNeasy Plant mini kit was followed. Briefly, a detergent buffer and RNase were added to the lyophilised material and incubated, resulting in cell membrane lysis. A precipitation solution was then added causing precipitation of detergents, proteins and polysaccharides. After centrifugation the supernatant containing the DNA was removed to a QIAshredder Mini spin column to remove most remaining precipitates and cell debris. An ethanol containing solution was added to the flow through before filtering this through a DNeasy Mini spin column, which binds the DNA. The DNA was washed twice with the supplied wash buffer containing ethanol. Elution buffer was added to the DNeasy column membrane allowing solubilisation of the DNA, followed by centrifugation to move the DNA into a clean microfuge tube. For full details see the DNeasy Plant Handbook (www.qiagen.com).

Assessment of Extraction Method Characteristics

Extensive assessment of the DNA extractions procedures was performed. A brief description of the methods appears below; however, individual experiments are addressed separately.

Extraction Efficiency

Extractions of small sub-samples of ground tissue were tested in an attempt to improve DNA extraction efficiency by decreasing sample size, for example from 40 mg to 5 mg.
After initial extraction, the original material underwent the extraction procedure again in order to determine whether total DNA extraction was occurring.

CTAB materials underwent additional back-extraction where 500 µL of TE buffer was added to the remaining organic phase after removal of the initial aqueous phase. The mixture was vortexed, incubated for 10 minutes at 65°C and centrifuged to allow removal of the aqueous phase. The aqueous phase also underwent re-extraction as previously described for the CTAB method.

Testing of extraction efficiency of the CTAB and Qiagen methods on pure wheat and *F. pseudograminearum* samples was also performed to check for bias for one type of tissue. In addition, known mixtures of either pure wheat and *F. pseudograminearum* tissues or pure wheat tissue spiked with different amounts of pure *F. pseudograminearum* DNA were also examined.

Inter-Sample Extraction Variation

Sample tissues were ground and combined into one large sample. This was then separated into individual sub-samples of similar weights and extracted to determine the variation between and within extraction methods.

DNA Quality Assessment

Five microlitres of each isolated DNA suspension was run on a 1.2% agarose gel at 90 volts for 30 minutes to determine the quality of DNA in the sample. Samples were visualised using a GelDoc system (BioRad, Gladesville, New South Wales, Australia). DNA in each sample was quantified (260nm wavelength) using a nanophotometer (Implen, Munich, Germany), with

absorbance readings (260/280nm ratio) also reporting contamination with RNA or carbohydrates/alcohols.

Agarose Gel DNA Quantification Method

- Add 1.56g agarose to 130mL of 1x TAE buffer
- Heat mixture until clear
- Cool mixture by running water over the bottle
- Add 1µL of ethidium bromide (10mg/mL)
- Pour mixture into mould and remove bubbles
- Set gel for approximately 1 hour
- Place gel into system
- Pour 330mL of water onto the gel
- Then pour 790mL of 1x TAE buffer into the well surrounding the gel
- Gently remove combs
- Add 5µL DNA + 4µL formamide/bromothymol blue loading buffer (formamide buffer) into each well
- Add 5µL of Standard DNA (Tomato DNA (100ng/ µL) + 4µL water + 5µL formamide buffer) mix into well at the end of the row
- Run gel for 30 minutes at 90 volts
- Gel is visualised using GelDoc
- Concentration of DNA is judged by brightness of visible band.

Experiment 1: Initial Extraction Technique Quality Assessment

Homogenous ground lyophilised wheat leaf tissue, coming from infected wheat seedlings, was measured into 60 microfuge tubes. The weight of the material in each tube was 40 mg (± 1 mg). Material was extracted using the Wizard Genomic DNA Extraction Kit, Qiagen DNeasy Plant extraction kit and the CTAB method. Each method was applied to 20 microfuge tubes. Within the set of 20, 10 were treated with RNase at the appropriate step and 10 underwent no RNase treatment. All DNA samples were eluted in 200 µL of autoclaved MilliQ water or Elution buffer where appropriate.

Results and Discussion

Wizard

Initial results demonstrated that Wizard extraction of 40 mg tissue samples did not yield DNA sufficient for downstream qPCR assessment on a consistent basis due to extracted DNA containing small pieces of cell debris and low DNA quality due to shearing. Use of lower sample weights for extraction may improve the quality of the Wizard extraction but as this was considered later during the experimentation period and due to the reported difficulties with the Wizard extraction, the Wizard method was not used for the major seedling experiments. Further testing on smaller samples is reported in Experiments 2, 3 and 6. These experiments demonstrate the usefulness of the Wizard technique for large scale extractions due to cost effectiveness and a simpler extraction procedure than the CTAB and Qiagen procedures.

RNA was present in all samples, but less when RNase treatment was applied. While DNA of pure material from wheat or *F. pseudograminearum* was of sufficient quality, infected LSs gave poor quality DNA. This DNA could be used in qPCR but methods giving better quality, cleaner DNA may improve reliability. The Wizard samples were not further assessed for DNA concentration in this experiment.

Qiagen

Qiagen extractions resulted in high quality DNA being extracted. Re-extraction of the original material resulted in further DNA being extracted. The initial extractions ranged between 150 and 30 ng/µL of DNA, with re-extractions containing approximately 15 ng/µL of DNA (Fig. 2C.1). All Qiagen initial samples were treated with RNase and no RNA was detected in samples after extraction.

CTAB

The CTAB extraction method resulted in high quality DNA being recovered, with limited shearing. RNA was only present in samples not undergoing RNase treatment. Back-extractions did not provide useful levels of DNA (Fig. 2C.1). However, re-extraction did result in further DNA being extracted. CTAB extractions performed with no RNase treatment yielded from 150-350ng/µL, while RNase treated samples gave 60-130ng/µL of DNA. Back-

extractions yielded from 0 to 8ng/µL DNA. Re-extractions with no RNase gave from 40-65ng/µL DNA, while RNase treated samples gave 25-50ng/µL of DNA.

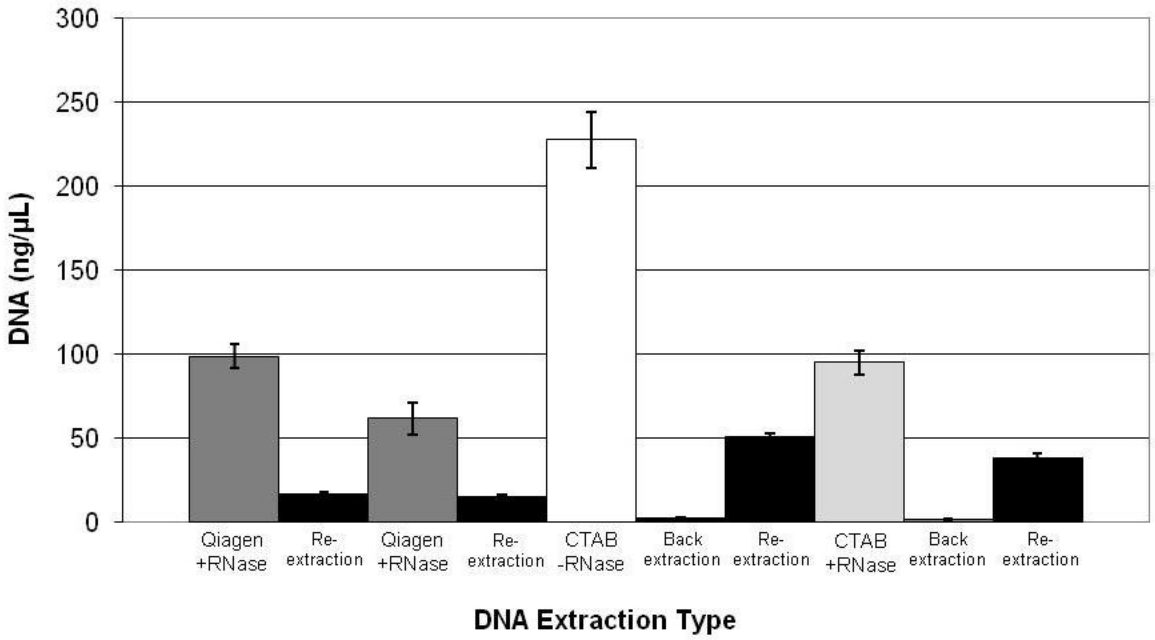

Figure 2C.1. Average quantity of DNA extracted across ten samples. The re-extraction and back extraction (for CTAB) DNA quantities for each set are shown to the right. Bars represent the standard error.

In terms of the goals of this experiment, to find a safe, repeatable DNA extraction method giving good quality DNA, the Qiagen and CTAB methods both gave satisfactory results, but improvements will be investigated further.

Total DNA extraction was not possible with these methods using 40 mg of dry material, as seen by the significant amounts of DNA present in re-extractions. RNase treatment appeared to result in a decrease in the amount of DNA extracted in the CTAB extracted samples, however, due to the importance of producing DNA samples of high purity for qPCR, the addition of RNase to the samples was considered too important to omit.

The CTAB, Wizard and Qiagen methods were further tested using smaller sample sizes to attempt to improve the efficiency of DNA extraction.

Experiment 2: Qiagen, CTAB and Wizard Extraction using 5 mg Samples

Homogenous lyophilised material of wheat leaves, coming from at least partially infected wheat seedlings, was measured into 15 microfuge tubes. The weight of the material in each tube was 5 mg (± 1 mg). Ten tubes containing 1 mg of dry material (± 0.02 mg) were also weighed out for extraction.

Material was extracted using the Qiagen Plant DNA extraction kit with 10 minutes incubation (1 and 5 mg), Qiagen Plant DNA extraction kit with 1 hr incubation (5 mg) and the CTAB method (1 and 5 mg). Each method was applied to 5 microfuge tubes. These samples underwent total re-extraction.

An experiment using only the CTAB method was performed on 40 mg dry samples. The incubation time was tested at 1 and 3 hours, containing either 1000 µL or 1500 µL of CTAB buffer.

A further set of 12 tubes containing 5 mg of homogenous lyophilised material was weighed out from a second tissue source. Ten of these tubes underwent Wizard extraction; the remaining two were extracted using the Qiagen kit.

All samples were eluted in 200 µL of autoclaved MilliQ water or Elution buffer where appropriate.

Results and Discussion

The 1 mg samples yielded DNA at levels too low to measure accurately using the nanophotometer. In the agarose gel the 1 mg bands of DNA could faintly be seen in the Qiagen extraction but were too faint in the CTAB extractions. One milligram was also difficult to accurately measure out before extraction.

The 5 mg samples extracted using Qiagen gave approximately 25ng/µL of DNA (Fig. 2C.2), with a 10 minute or 1 hour incubation having no effect on the efficiency of DNA extraction. The bands of DNA in the agarose gel also appeared similar, with no degradation. The CTAB method yielded more DNA from 5 mg (~35ng/µL) than Qiagen but was degraded when observed in agarose. Re-extraction in all cases yielded more DNA.

Variants on the CTAB method gave poor quality DNA. The original method gave the best results, but DNA degradation still occurred.

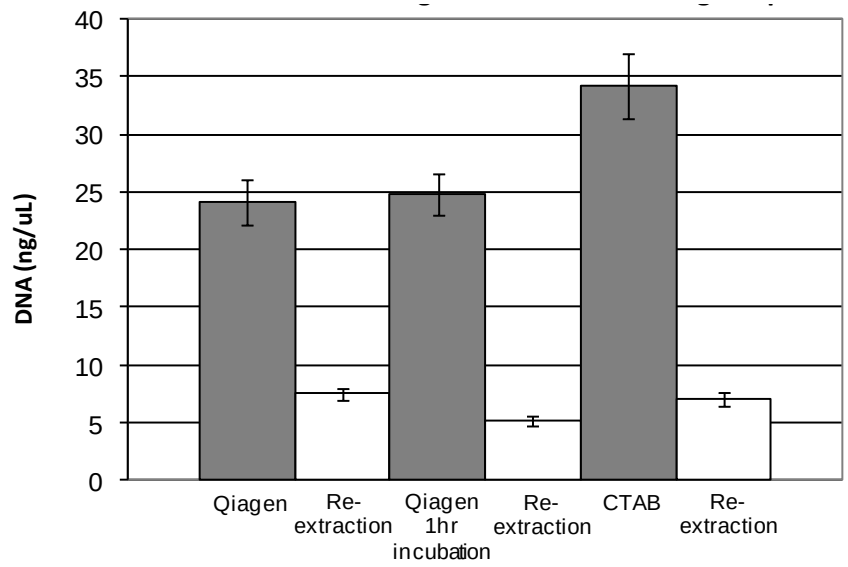

Figure 2C.2. DNA quantities extracted from 5 mg samples using the Qiagen method, with either 10 min or 1 hr incubation, or the CTAB method. Re-extracted DNA quantities are shown to the right. Bars represent the standard error.

The Wizard method gave moderate to good quality DNA when observed in an agarose gel. The concentrations of DNA extracted using Wizard were higher than Qiagen (Fig. 2C.3), however, the difference was less when compared to the difference between CTAB and Qiagen.

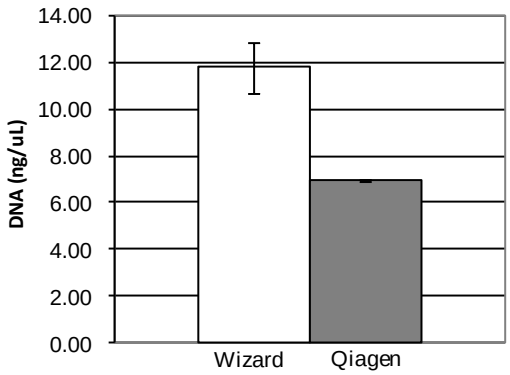

Figure 2C.3. DNA quantities extracted from 5 mg samples using the Wizard and Qiagen methods.

Simply, Qiagen gave better quality DNA, albeit at lower levels than CTAB and Wizard. None of the methods allowed for total re-extraction. The use of 5 mg sub-samples for extraction appears to be acceptable as the standard error is small. In terms of qPCR, the 5 mg samples also gave DNA concentrations satisfactory for immediate use in the PCR reaction.

The next step was to quantify the samples using qPCR to examine the normalised values achieved for these Qiagen, CTAB and Wizard extracted samples.

Experiment 3: DNA Sample Analysis Using Quantitative PCR

The RNase treated 40 mg samples from Experiment 1 and the 1 mg and 5 mg (10 minute incubation) samples from Experiment 2 were run in the qPCR assay (described in Chapter 2) in order to compare efficiencies of CTAB and Qiagen, and Wizard and Qiagen. All samples within each test originated from the same homogenous tissue source, and thus should have similar normalised values. Normalised values were calculated by dividing the *F. pseudograminearum* DNA quantity by the wheat DNA quantity (ng *Fp* DNA / ng Wh DNA). The Qiagen/CTAB and Qiagen/Wizard comparison tests had different tissue sources.

Results and Discussion

The normalised values of the CTAB extractions were all between 0.03 and 0.04, showing consistency between sample sizes, with small standard errors within groups (Fig. 2C.4). The Qiagen samples gave higher readings between 0.045 and 0.07, showing a greater range than the CTAB samples. Again, the standard errors were consistently small.

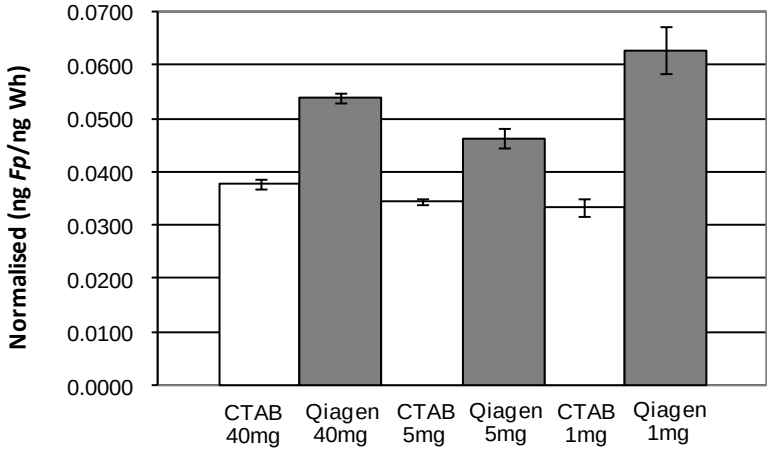

Figure 2C.4. Normalised DNA values for Qiagen and CTAB extractions from samples of different weights. Bars represent the standard error.

The normalised values of the Wizard extractions were between 0.025 and 0.03 (Fig. 2C.5). The Qiagen samples gave only slightly higher readings between 0.03 and 0.035. The standard errors again were consistently small. Compared to the CTAB method, the Wizard extraction method produced results more consistent with the Qiagen method when using 5 mg samples.

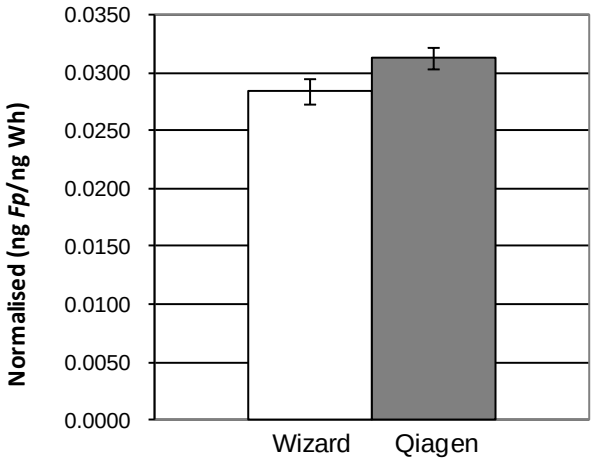

Figure 2C.5. Normalised DNA values for Qiagen and Wizard 5 mg extractions. Bars represent standard error.

From these results the CTAB method appeared to produce the most reliable results when extracting different tissue weights. However, the degradation of DNA in the CTAB method was a concern, particularly for samples which may be heavily infected with *F. pseudograminearum* and thus be more fragile. The effect of degraded DNA on PCR results is difficult to examine, but eliminating this as a potential problem was the best option. Therefore, Qiagen, even though demonstrating less consistency between different weight samples than CTAB, was still considered to be the most reliable method. Another experiment to determine any bias in the extraction procedure was performed in order to determine if the methods extract the host or pathogen DNA with different efficiencies. The Wizard method demonstrated results similar to the Qiagen extractions when using 5 mg extraction weights and while more care must be taken with this procedure to avoid contamination with cell debris, Wizard should be considered as a cheap and rapid alternative to Qiagen extractions.

Experiment 4: Qiagen and CTAB Extraction of Wheat and *F. pseudograminearum* Samples

Samples of pure *F. pseudograminearum* mycelium were extracted to produce a standard curve of dry weight of mycelium compared to amount of DNA extracted. Pure wheat and combined samples of *F. pseudograminearum* and wheat were also extracted (Table 2C.1). All extractions included five replicates of each sample type for both Qiagen and CTAB methods.

Table 2C.1. Samples types used to compare DNA extraction efficiencies.

Sample Types	Reference Code
Fp 1 mg	Fp1
Fp 2.5 mg	Fp2.5
Fp 5 mg	Fp5
4 mg Wh + 1 mg Fp	Wh4Fp1
5 mg Wh	Wh5
5 mg Wh + 200ng Fp DNA	Wh5+200Fp

DNA quantities were measured using both the nanophotometer and qPCR. Each sample was measured twice on the nanophotometer and then the average of the 5 samples was calculated. In the PCR reaction each sample was run in duplicate.

Results and Discussion

The DNA quantities were more consistent and had less shearing for Qiagen extractions of all sample types. Extracted DNA quantities for each method and sample, determined using both the nanophotometer and qPCR, and differences between the two methods are shown in Figures 2C.6, 2C.7 and 2C.8, respectively.

The differences in the CTAB method between pure *F. pseudograminearum* and wheat 5 mg extractions using the nanophotometer were a concern as such a difference in DNA yield was not expected. This concern was deepened by the great difference between CTAB and Qiagen for the pure *F. pseudograminearum* samples, where CTAB yielded approximately double for each of the three sample sizes. The similarity then between CTAB and Qiagen for the pure wheat samples and even the mixed samples is closer to the expected outcome.

Overall, the CTAB method was inconsistent. This is demonstrated by the differences observed between nanophotometer and qPCR readings (Fig. 2C.8). However, the consistency of extractions of the 5 mg samples of pure and mixed materials produced by Qiagen demonstrated it to be a more reliable method. Even though differences between nanophotometer and qPCR readings were still evident for the Qiagen extracted samples, overall they were smaller for all sample types.

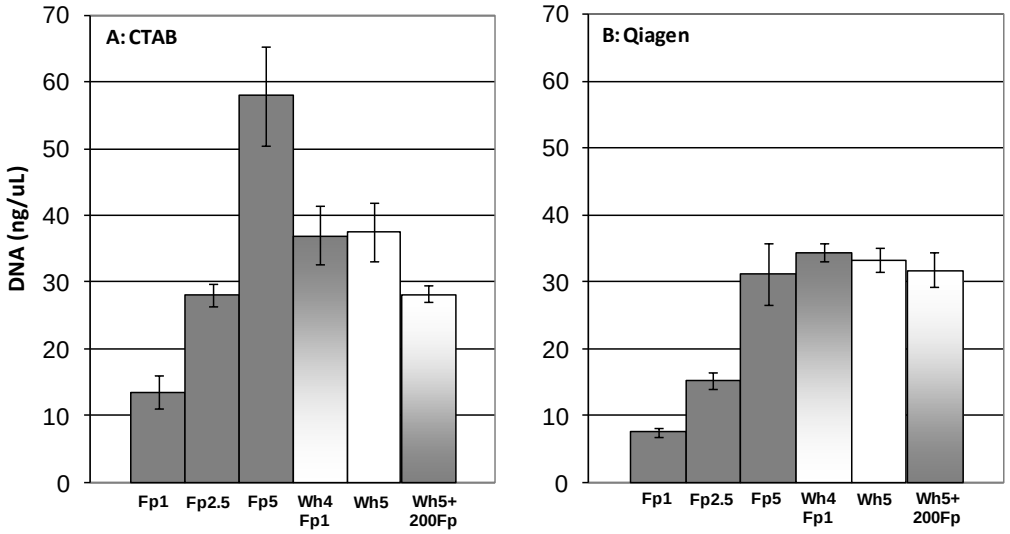

Figure 2C.6. DNA extraction yields of various samples types determined via nanophotometer. A. CTAB. B. Qiagen. Bars represent the standard error.

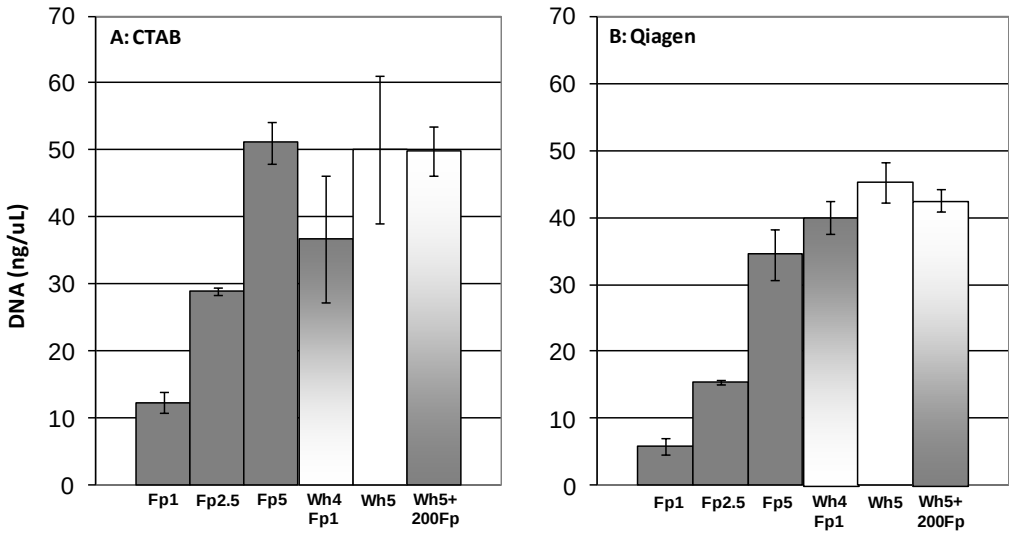

Figure 2C.7. DNA extraction yields of various samples types determined via qPCR. A. CTAB. B. Qiagen. Bars represent the standard error.

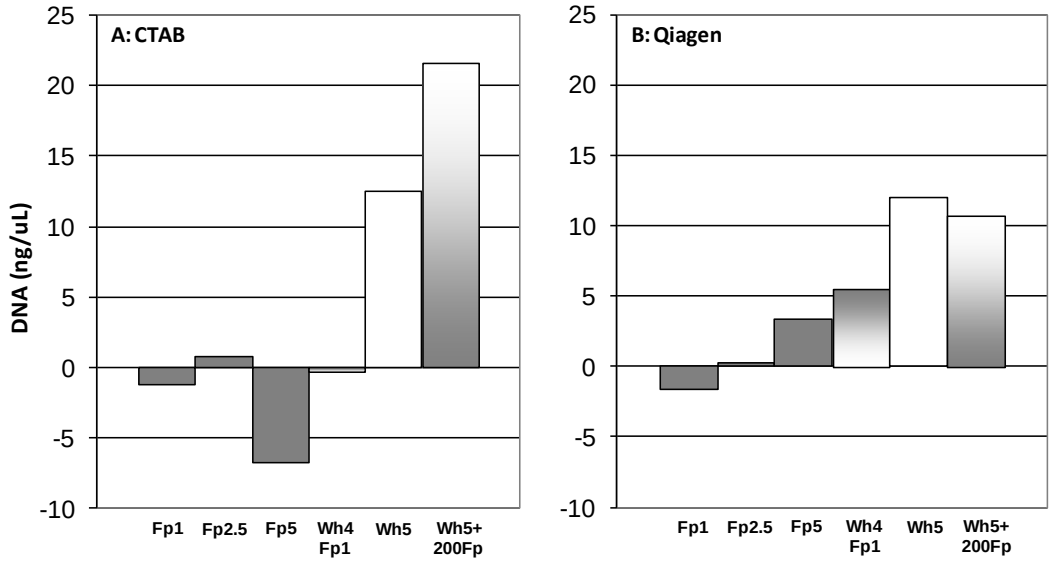

Figure 2C.8. DNA quantity differences between nanophotometer and qPCR readings. Differences are the qPCR values minus the nanophotometer values.

The two combined sample types, Wh4Fp1 and Wh5+200Fp, had normalised ratios calculated using qPCR data (Tables 2C.2 and 2C.3). Expected ratios were calculated using the DNA quantity, as determined by qPCR, from pure samples. The quantities of *F. pseudograminearum* DNA present in each sample were also compared (Fig. 2C.9). The comparison of the *F. pseudograminearum* quantities in the two mixed samples between CTAB and Qiagen demonstrated that each method gave similar mean results. However, the Qiagen method gave much smaller standard errors than the CTAB method. The normalised values demonstrated that the Qiagen samples gave more predictable results than CTAB for the Wh4Fp1 samples. The inaccuracy of the Wh5+200Fp samples, showing about a ten-fold difference from expected to actual, is suggested to be due to experimental error, specifically because of incorrect quantitation of the initial *F. pseudograminearum* DNA solution added to the 5 mg of wheat material.

Table 2C.2. Expected and actual normalised values for sample type Wh4Fp1 using Qiagen and CTAB extractions.

Wh4Fp1	Qiagen	CTAB
Expected	0.175	0.283
Actual	0.139	0.148

Table 2C.3. Expected and actual normalised values for sample type Wh5+200Fp using Qiagen and CTAB extractions.

Wh5+200Fp	Qiagen	CTAB
Expected	0.022	0.020
Actual	0.003	0.003

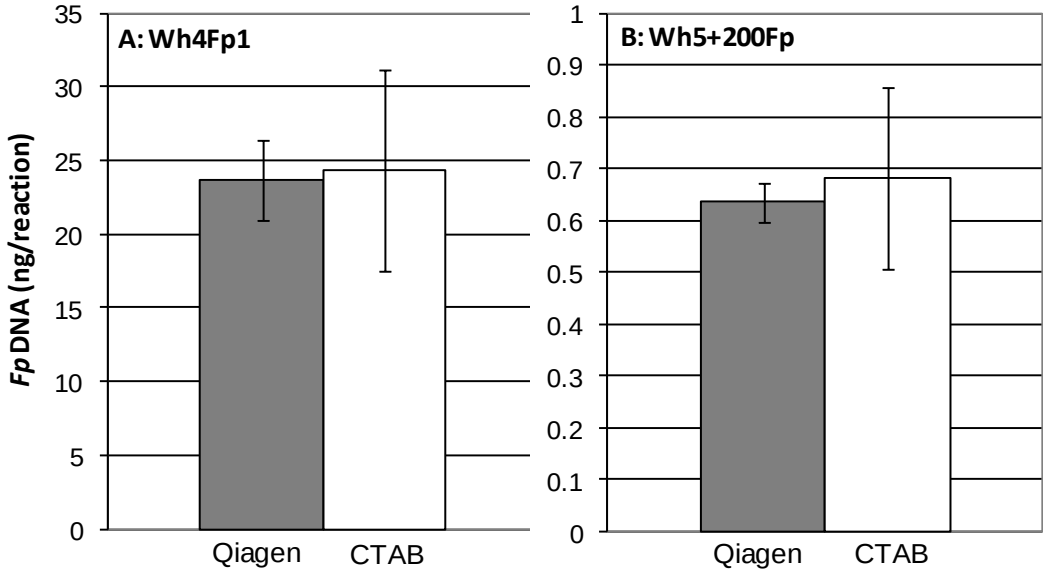

Figure 2C.9. *F. pseudograminearum* DNA quantities in combined samples determined via qPCR. Bars represent the standard error.

Strong linear relationships were observed between pure *F. pseudograminearum* mycelium dry weights and extracted DNA for both extraction procedures (Fig. 2C.10).

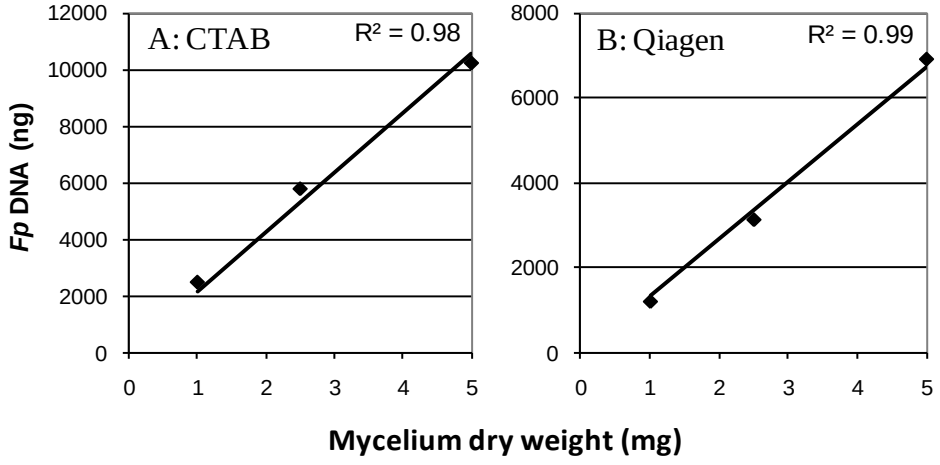

Figure 2C.10. Linear relationships between *F. pseudograminearum* DNA and mycelium dry weights for CTAB (A) and Qiagen (B) extracted samples.

In conclusion, the CTAB and Qiagen methods did not reveal a great deal of dissimilarity between results, certainly not enough to show a clear choice of the better extraction method. However, due to the consistently smaller standard errors and better DNA quality of the Qiagen extractions, and the lack of hazardous chemicals combined with the shorter extraction time, Qiagen DNA extraction was the preferred method in later experiments. One final experiment to demonstrate the accuracy of this method was performed.

Experiment 5: Qiagen Sample DNA Spiking

This experiment used a range of different tissues to assess DNA extraction efficiency (Table 2C.4). Briefly, 25 microfuge tubes containing 5 mg of pure wheat tissue were divided into 5 groups. A previously extracted *F. pseudograminearum* DNA sample was quantified to contain 63ng/µL of DNA using the nanophotometer. Four of the groups of 5 tubes were spiked with a known amount of DNA before extraction. Five control tubes of the pure wheat tissue were also extracted and quantified to determine how much DNA was in 5 mg of the wheat tissue.

Pure wheat materials were also spiked with *F. pseudograminearum* mycelium and infected wheat material. The quantity of *F. pseudograminearum* in the infected material was previously calculated using qPCR. The contents of the samples are described in Table 2C.4. Expected normalised ratios were compared with the actual ratios.

Table 2C.4. Tissue samples used to evaluate the extraction efficiency of the Qiagen method.

Sample Code	**Wheat Tissue (mg)**	**Infected Wheat Tissue (mg)**	***Fp* Mycelium (mg)**	***Fp* DNA (ng)**
Wh5	**5**	**-**	**-**	**-**
Wh5/315	**5**	**-**	**-**	**315**
Wh5/630	**5**	**-**	**-**	**630**
Wh5/1260	**5**	**-**	**-**	**1260**

Wh5/2520	**5**	**-**	**-**	**2520**
Wh4+Fp1	**4**	**-**	**1**	**-**
Wh4+I1	**4**	**1**	**-**	**-**
Wh3+I2	**3**	**2**	**-**	**-**

Results and Discussion

Actual normalised values were consistent with the expected normalised values for all sample types. Starting material did not have a significant effect on the normalised value observed as either pure wheat tissue spiked with *F. pseudograminearum* DNA (Figure 2C.11), combined pure wheat and *F. pseudograminearum* samples (Figure 2C.12) or pure wheat mixed with infected wheat (Figure 2C.13) all demonstrated consistency with expected normalised values. The results were also consistent across a large range of normalised values (0.0004 → 0.25). The normalised values detected demonstrate the sensitivity of the extraction method, allowing very small quantities of *F. pseudograminearum* to be extracted while in the presence of wheat tissue. In addition, the inter-sample variability was low, as proven by the predominantly small standard errors.

The Qiagen extraction kit produced reliable quantities of DNA of both wheat and *F. pseudograminearum* while extracting different combinations of each organism. These results, along with the previous results for DNA extractions provide enough positive evidence for the Qiagen kit extraction method to be suitable for the experiments performed within this project.

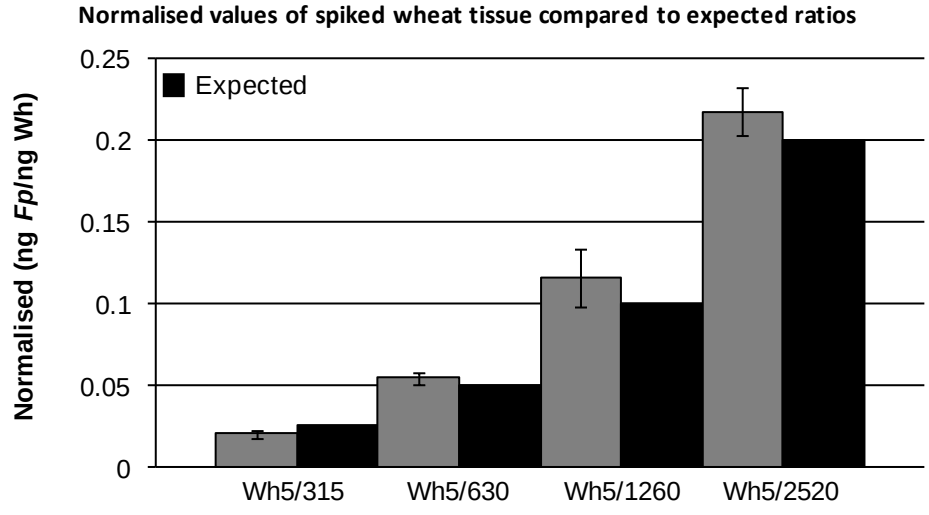

Figure 2C.11. Normalised values for samples spiked with *F. pseudograminearum* DNA. Expected values are shown to the right. Bars represent the standard error.

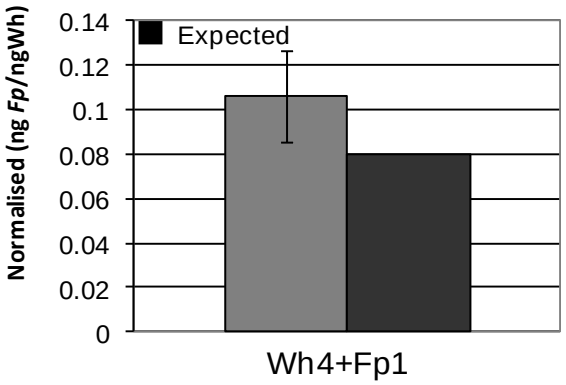

Figure 2C.12. Normalised values for samples containing a combination of 4 mg of pure wheat tissues and 1 mg of pure *F. pseudograminearum*. Expected value is shown to the right. Bars represent the standard error.

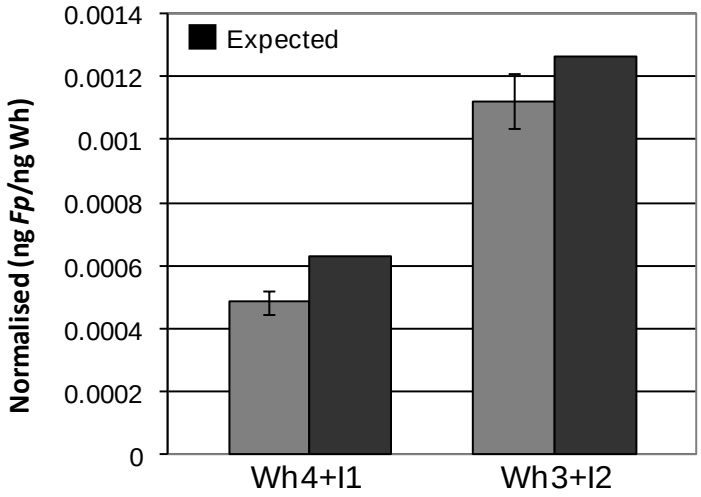

Figure 2C.13. Normalised values for samples containing a combination of pure wheat and infected wheat tissues. Expected values are shown to the right. Bars represent the standard error.

Experiment 6: Assessment of Wizard Extraction for Adult Plant Tissue

During the project an experiment comparing tissues of field grown adult wheat plants was attempted. This was calculated to require a significant amount of extractions. As the Qiagen extraction method was expensive and the quality of DNA in adult plant tissues was dubious,

the Wizard method was considered as an alternative. A brief comparison of the two extraction methods, using infected internodes, was performed.

Ten tubes containing 15 mg (± 1 mg) of ground, lyophilised, infected internode tissue were assessed, five using the Wizard method and five using the Qiagen method. A comparison of the normalised qPCR results was performed to assess the two methods.

Results and Discussion

The normalised qPCR values were similar between the Wizard and Qiagen extracted samples (Fig. 2C.14). From this result it was determined that the Wizard extraction method would be used for experiments extracting DNA from adult tissues. This allowed for cheaper and more rapid DNA extractions. It must be noted that care had to be taken with the Wizard extracted samples to produce consistent results, particularly during the first centrifugation step. This step was increased from 5 to 10 min to allow better removal of small pieces of cell debris.

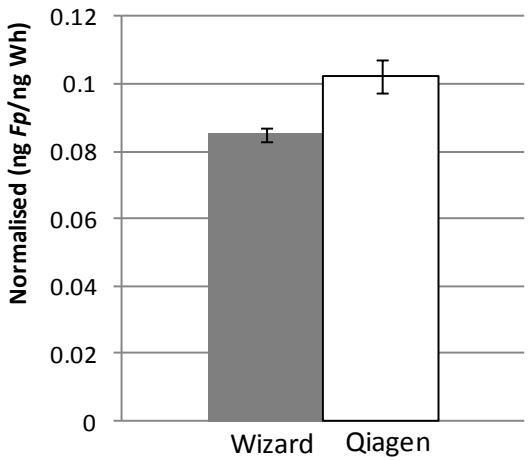

Figure 2C.14. Normalised values for adult plant tissues extracted using the Wizard and Qiagen methods. Bars represent the standard error.