

A HIGH THROUGHPUT, NON-ORGANIC METHOD FOR PLANT GENOMIC DNA ISOLATION

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Abstract

A simple and reliable method for extracting genomic DNA from leaf and seed of monocotyledonous and dicotyledonous plants and ascomycete fungal mycelium is described. The method requires only 50mg of sample and yields approximately 10µg of high quality DNA. It involves inexpensive, non-organic constituents and can reliably be used for the parallel isolation of 384 DNA samples suitable for PCR based marker analysis. The yield and high quality of extracted DNA from different species, combined with the use of inexpensive, non-hazardous reagents, provides a cost-effective method compatible with a 96 well format. The resultant DNA is suitable for PCR and subsequent fragment analysis using capillary electrophoresis.

Introduction

Marker assisted selection is of increasing importance in plant breeding programs, where large numbers of genotypes are screened for numerous traits in a short time period. While the availability of DNA markers linked to traits of interest has increased, available DNA extraction procedures have limited the number of samples able to be processed. Most of the DNA extraction methods often require the use of hazardous chemicals and/or are inconsistent in terms of reliably producing sufficient quantities of high quality DNA (Dellaporta *et al.*, 1983; Guillemaut & Marechal-Drouard, 1992; Marechal-Drouard & Guillemaut; 1995, Štorchová *et al.*, 2000). Methods based on commercially available kits or their modifications enhance the quality and quantity of extracted DNA but are generally not cost-effective for large-scale marker screening (Doulis *et al.*, 2000). Several researchers have attempted to eliminate the use of hazardous chemicals, expensive kits, equipments and labour-intensive steps for high throughput DNA extraction. However, the DNA isolated using these techniques generally has a limited shelf life, is of a purity that can only be used with certain markers, can only be recovered in small quantities sufficient for PCR analysis, can amplify only small fragments due to its damaged nature, and is reliable only when used at a certain dilution (Dilworth & Frey, 2000; Ikeda *et al.*, 2001, Saini *et al.*, 1999, Klimyuk *et al.*, 1993, Lin *et al.*, 2000, Paris & Carter, 2000,). Some of these methods use acid and alkali treatments to generate DNA template that require very stringent conditions to amplify only specific DNA sequences using PCR (Klimyuk *et al.*, 1993; Paris & Carter, 2000). In the present study we have described a simple alkali based method to recover high quality double stranded DNA that can be used for a range of molecular biology applications.

Materials and Methods

Plant, seed and fungal material: Leaves (50mg) were harvested from 4-21 day-old wheat (*Triticum aestivum* L.), lupin (*Lupinus albus* L.), canola (*Brassica napus* L.) and barley (*Hordeum vulgare* L.) seedlings. Each leaf sample was divided into 3-4 equal pieces, transferred into 1.2ml tubes in a 96 well format (Qiagen Pty Ltd., Australia), containing two 3mm stainless steel balls. Samples were stored frozen at -80°C prior to

use. Single barley and wheat seeds were ground to a fine powder, using a hammer and collected in the same 96 well format.

Mycelia from the ascomycete fungi *Botryosphaeria lutea*, was obtained by inoculating 15ml of potato dextrose broth (PDB, Difco Laboratories, Detroit, MI, USA) with a 0.5cm plug from a five-day-old agar culture. The broth culture was then incubated at 25°C for five days in darkness, on a rocking platform. The resulting mycelial growth was removed from PDB and washed with MQ H₂O. The agar-plug was removed and the mycelia blotted-dry on sterile paper towel. Samples (50mg) were then placed in 2ml tubes and frozen in liquid nitrogen, before being stored at -80°C.

DNA extraction: A new method (Method 2) was evaluated for ability to extract DNA from a range of species. DNA was isolated from wheat, canola, lupin and *B. lutea* using the described method, a modification of the described method, a modification of the method of Štorchova *et al.*, (2000), the standard phenol chloroform method (Davies *et al.*, 1986) (Table 1).

Unless mentioned otherwise, the DNA was extracted using the following protocol:

Solutions and reagents

Extraction Buffer (EB): 0.05M Tris-HCl, 0.3M EDTA pH 8.0
10% Sodium dodecyl sulphate (SDS)
4.2M Sodium perchlorate (NaOCl₄)
0.2M Sodium hydroxide (NaOH).
3M Sodium acetate pH 5.5 (NaOAc)
Tris-EDTA buffer (TE): 10mM Tris-HCl, 0.1mM EDTA, pH 8.0
Ethanol (Absolute)
70% ethanol (v/v)
Liquid Nitrogen

- Remove 2×96 well, 1.2ml micro tubes containing leaf or flour samples from -80°C freezer and immerse in liquid nitrogen for 5 min. Prevent samples from thawing at all times.
- Pulverise the samples to a fine powder by shaking for 60-90 seconds in a Qiagen mixer mill (Qiagen Pty Ltd, Australia). Briefly spin the samples to collect the powdered leaf material in the bottom of the tube. This step is not required for flour samples.
- Add 220µl of EB and 30µl of 10% SDS, and incubate at 37°C for 15 min. A multi-channel pipette can be used to aliquot EB and all subsequent solutions.
- Add 150µl of 0.2M solution of NaOH and 150µl of 4.2M NaOCl₄. Mix well for 15 min., under constant shaking. Chill at -20°C for 5-10 min., before centrifuging at 4°C for 15 min.
- Transfer 250µl of the supernatant to fresh 1.2ml tubes.
- Precipitate the DNA with 25µl of NaOAc and 500µl of absolute ethanol.
- Centrifuge at room temperature for 15 min. Discard the supernatant and wash the DNA pellet with 1ml of 70% ethanol. Discard the 70% ethanol. Remove any residual ethanol by inverting the tubes on a piece of paper towel for 5 min.
- Dry the pellets under vacuum for 5 to 10 min., do not over dry. Dissolve the DNA in 50µl of TE buffer and store at -20°C.
- To obtain DNA free of RNA, add 2µl of 10mg/ml RNase-A to the sample and incubate for 30 min., at 37°C.

Separate 1-2µl of the DNA on a 1.0 % TAE agarose gel to observe the integrity of DNA.

Different combinations of Proteinase-K (20 μ g), 0-0.58M Sodium hydroxide (NaOH), 0-1.15% Sodium hypochlorite (NaOCl) and 0-4.2M Sodium perchlorate (NaOCl₄) with and without phenol-chloroform extraction were used to determine their effects on DNA isolation efficiency and reliability. Each combination always included 220 μ l of Extraction Buffer (EB) and 30 μ l of 10% SDS added at step 3 of the DNA extraction protocol. DNA isolated employing a phenol-chloroform extraction method (Davis *et al.*, 1986) was conducted essentially as described above, with the exception of the addition of 20 μ g of Proteinase-K to the extraction buffer and at step 4, NaOCl₄ and NaOH were replaced with 150 μ l each of phenol and chloroform. This was used as a standard for comparison to the DNA extracted using the described procedure.

DNA quality was examined following electrophoresis on agarose gels. Spectrophotometric analysis was performed on duplicate samples of extracted DNA. Absorbance was recorded at A260, A280 and A320 nm using Eppendorf BioPhotometer Model 6131, (Eppendorf AG, Hamburg, Germany), with each reading recorded in triplicate. The A260/A280 ratio was calculated and a mean taken for each sample.

Restriction analysis and electrophoresis: Digestion of DNA samples, using enzymes *Bam*H1 and *Eco*R1, was performed according to manufacturer's specifications (New England Biolabs, MA, USA). One microgram of digested and undigested genomic DNA, together with 0.5 μ g of pGEM^R DNA Markers (Promega Corporation, Michigan, WI, USA) or TrackitTM 100 bp DNA ladder (Invitrogen, USA) were separated on a 1.0% TAE agarose gel at 80 Volts for 2 hrs, stained with Ethidium bromide and viewed under UV illumination.

PCR amplification and fragment analysis: PCR was performed in 12 μ l reaction volume containing 80ng DNA, 10ng of forward primer tagged with a M13 generic sequence, 30ng of M13 core sequence labelled with one of three fluorescent dyes D2, D3 or D4 (Beckman Coulter Inc, Fullerton, California), and 20ng of unlabelled reverse primer, 25 μ M dNTPs, 2mM MgCl₂ and 1.0 unit of *Taq* DNA Polymerase (Promega Corporation, Michigan, WI, USA). For vertical PAGE analysis, 0.5 μ M each of untagged and unlabelled forward and reverse primers were used in the amplification reaction. Reactions were cycled in an ABI GeneAmp 2700 PCR System (Applied Biosystem, California, USA). Primer sequences and PCR reaction conditions for primers *Xwm*c276 and *Xwm*c050 were obtained from the National Wheat Molecular Marker Program (NWMMMP) microsatellite database (<http://www.scu.edu.au/research/cpcg>) while primer *Xbm*ag353 nucleotide sequence, PCR reaction conditions and PAGE conditions used were as described by Raman *et al.*, (2003). Denaturing capillary electrophoresis and fragment sizing was performed using a CEQ 8000-Genetic Analysis System and associated software (Beckman Coulter Inc, Fullerton, California) as described previously (Raman *et al.*, 2005).

Results and Discussion

The objective of this investigation was to develop a high throughput DNA isolation method suitable for the molecular analysis in a range of species. The described method involves NaOH and can be used to isolate high quality genomic DNA from wheat, barley, canola, lupin and the fungus *Botryosphaeria*. This method could be implemented in a 96

well format and is suitable for high throughput screening platforms. One person can easily isolate DNA from 384 samples in 6-7 hrs. The average DNA yield is approximately 10 μ g per 50mg fresh sample tissue. Restriction digestion using *Bam*H1 and *Eco*R1 and an observed UV absorbance ratio (A_{260}/A_{280}) of approximately 2.0 indicated its suitability for an array of applications such as restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP) and PCR based marker assays. Fig. 1 shows the consistent recovery of good quality wheat DNA and its cleavage with restriction enzymes.

While determining the final protocol for DNA isolation, different combinations of Proteinase-K, NaOH, NaOCl₄ and NaOCl, with and without phenol-chloroform extraction, were also assessed. Fig. 2 shows DNA restriction digest using different combinations. The use of phenol-chloroform in the extraction yielded more DNA. However, DNA isolated using any combination that contained NaOH, with or without phenol-chloroform, was of better quality as suggested by complete restriction of the DNA. Sodium hypochlorite, when used alone in phenol-chloroform extraction, had no effect on DNA quality despite an observed bleaching effect on green chlorophyll pigments. DNA extracted with NaOCl₄ alone failed to undergo complete digestion with the enzyme *Bam*H1. However, Sodium perchlorate was added to the extraction mixture in the described protocol only to denature enzymes and provide protection against degradation of the DNA.

Different concentrations of NaOH were tried to determine optimum concentration for the recovery of non-denatured DNA. NaOH concentration ranging from 0.03M to 0.1M were sufficient for the recovery of high molecular weight DNA, that underwent complete restriction with enzyme *Bam*H1 and was suitable for subsequent PCR amplification. No intact high molecular weight DNA and corresponding amplification products were observed at the concentrations higher than 0.1M. These results indicate destruction of the DNA at higher concentrations of NaOH (Fig. 3a & 3b).

The size of PCR products generated using the extracted DNA was also assessed to determine if the extraction method had any effect on the integrity of the amplification product. DNA extracted from both seed and leaf samples using the described method amplified products of expected sizes for each marker, when used as a template for PCR (Fig. 4).

Table 1 compares the described method with and without sorbitol with two methods commonly used in different laboratories. Sorbitol was included to improve the resulting interface following the first round of centrifugation. Chloroform: isoamyl alcohol step was used to determine any effect on the removal of contaminants. Besides the use of inexpensive and non-hazardous chemicals the purity of the isolated DNA is comparable to methods that employ costly and hazardous chemicals such as CTAB, polyvinylpyrrolidone (PVP), phenol and chloroform. The DNA purity was judged by the A_{260}/A_{280} ratio which ranged from 1.92 to 2.39 across all samples, and its ability to be completely digested by restriction enzymes, suggesting no contaminating proteins and polysaccharides (Table 2). For wheat, extraction method 1 gave an unusually high A_{260}/A_{280} ratio (3.26) as did lupin for extraction method 2 (3.03). In both instances, this could be explained by the intense grinding procedure to which all samples were exposed resulting in unusually high UV absorbing contaminants with no adverse affect on the DNA quality. Optimisation of grinding times and ball bearing size would enable laboratories to customize the procedures to obtain ideal extraction conditions for different tissue types.

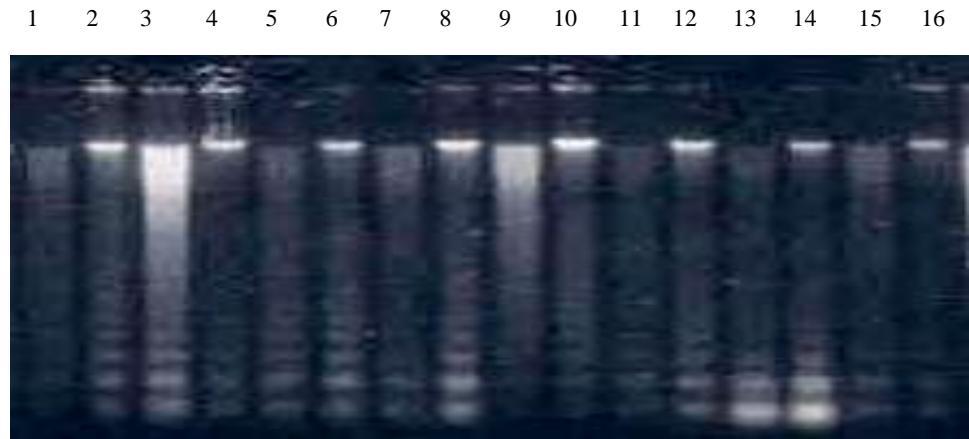


Fig. 1. Agarose gel electrophoresis of DNA samples from different wheat varieties restricted with *Bam*H1 (odd numbered lanes) and corresponding samples (even numbered lanes)

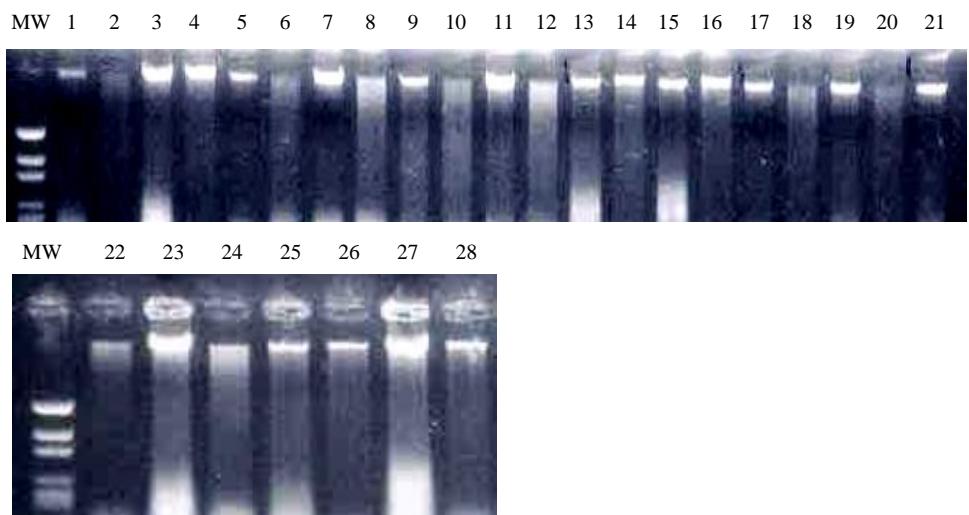


Fig. 2. Agarose gel electrophoresis of extracted wheat DNA using different combinations. Unrestricted DNA (odd numbered lanes) and *Bam*H1 restricted DNA (even numbered lanes). Lanes 1-4, NaOCl₄+PC; Lane 5-8, NaOCl₄+NaOH+NaOCl+PC; Lanes 9-12, NaOH+NaOCl+PC; Lanes 13-16, PC only; Lanes 17-20, NaOCl₄+NaOH+NaOCl. Lane 21-24, NaOH+NaOCl and Lanes 25-28, NaOCl₄ only. MW, pGEM^R Molecular Wt Marker.

As expected DNA preparation with phenol-chloroform method yielded more DNA, with consistently higher values at A_{260} , that appeared partially digested on agarose gel suggesting insufficient amount of enzyme *Eco*R1 that is required to fully digest the DNA; thereby imposing a need to employ an extra dilution step to reach optimal concentration for restriction digestion (Fig. 5).

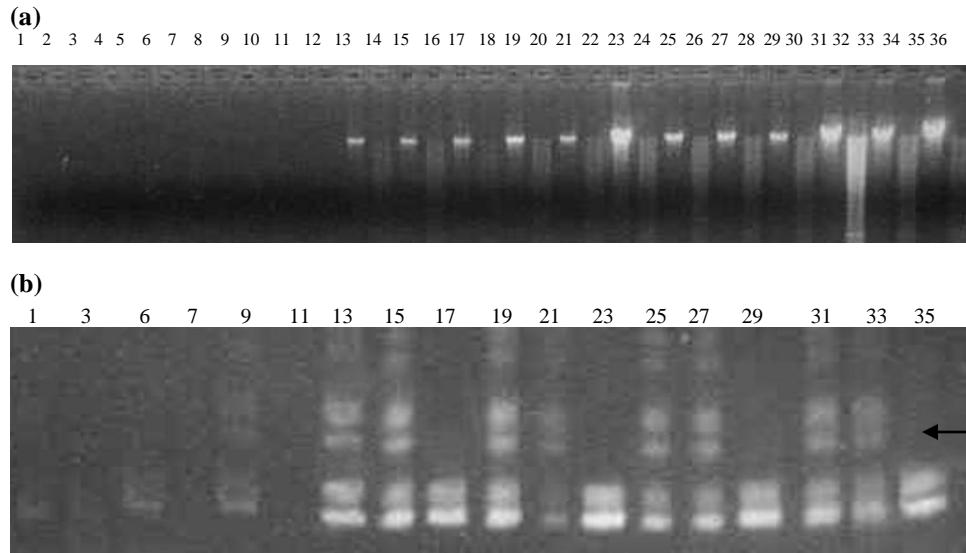


Fig. 3. (a). Agarose gel electrophoresis of *Bam*H1 restricted (even numbered lanes) and unrestricted DNA (odd numbered lanes). The DNA was extracted from barley lines using the described method using different concentration of NaOH. Lanes 1,3,5 0.28M NaOH; Lanes 7,9,11 0.2M NaOH; Lanes 13,15,17 0.1M NaOH; Lanes 19,21,23 0.06M NaOH; Lanes 25,27,29 0.03M NaOH; Lanes 31,33,35 Standard phenol-chloroform method. (b) corresponding PCR amplification of the unrestricted DNA (odd numbered lanes in panel a) using marker *Xbmag353*. Arrow indicates expected amplicon derived from barley variety Dayton.

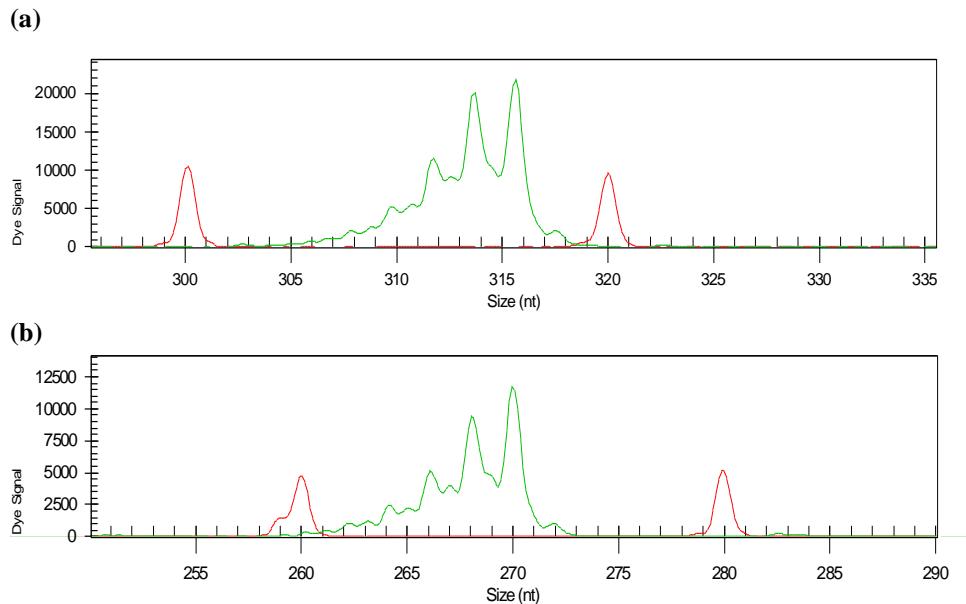
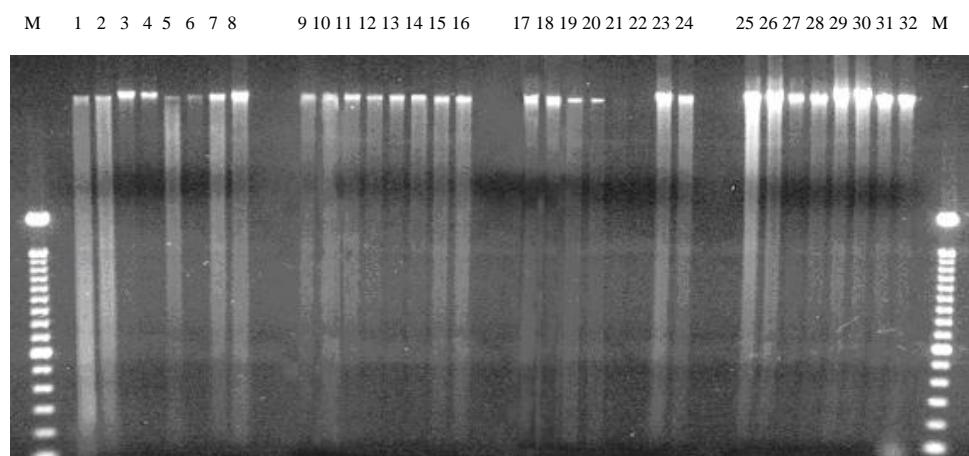


Fig. 4. Capillary electrophoresis of amplified fragments. DNA extracted using the described method (Method 2) was used as a template for PCR with fluorescently labelled primers. *Xwmc276c* used to amplify DNA extracted from leaf of cv Katunga (a). *Xwmc050* was used to amplify DNA extracted from seed of cv Sunbri (b). The amplicons were analysed on a Beckman CEQ 8000 genetic analyser. Red peaks are internal size standards.

(a)



(b)

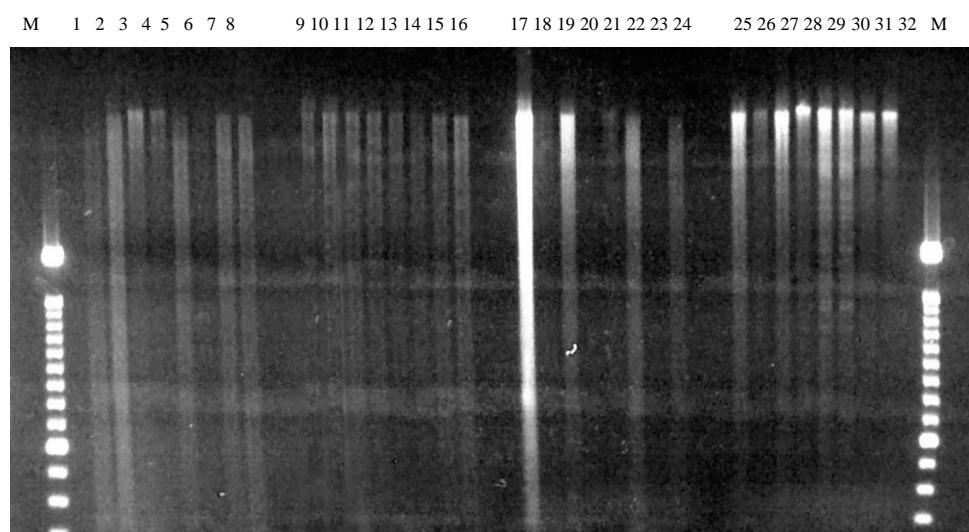


Fig. 5. (a) Agarose gel electrophoresis of DNA extracted from various samples using four extraction methods. (b) DNA samples were digested with restriction enzyme *Eco*R1. Lanes 1-8, extraction method 1. Lanes 9-16, extraction method 2. Lanes 17-24, extraction method 3. Lanes 25-32, extraction method 4. Wheat samples, lanes 1,2,9,10,17,18,25,16. Canola samples, lanes 3, 4, 11, 12, 19,20,27,28. Lupin samples, lanes 5, 6, 13, 14, 21, 22, 29, 30. *Botryosphaeria* samples, lanes 7, 8, 15, 16, 23, 24, 31, 32. M, Trackit™ Molecular Weight marker.

The significant finding of this investigation is the suitability of NaOH as a key ingredient, that has not been previously reported for the extraction of high quality double stranded DNA from a range of crop species. The procedure is amenable to high throughput applications and the resultant DNA is suitable for a range of techniques including RFLP and AFLP. Unlike other methods that require several expensive reagents,

the described method involves low-cost reagents such as sodium hydroxide and sodium perchlorate. Furthermore, the method eliminates the need for a fume-hood as it involves non-hazardous, non-organic components. The simplicity of the protocol also makes it amenable to scale up or down as required.

Table 2. Mean A260/A280 ratios for DNA extracted from different tissue types, using four extraction methods. Readings of duplicate samples were taken in triplicate and used to create the mean. Standard deviations are given in parenthesis.

| | Extraction method | | | |
|-----------------------|-------------------|-------------|-------------|-------------|
| | 1 | 2 | 3 | 4 |
| Wheat | 3.26 (1.24) | 2.09 (0.15) | 1.79 (0.05) | 1.99 (0.11) |
| Canola | 2.29 (0.30) | 3.03 (1.54) | 2.54 (0.88) | 2.01 (0.03) |
| Lupin | 2.19 (0.21) | 2.01 (0.13) | 1.55 (0.08) | 1.83 (0.05) |
| <i>Botryosphaeria</i> | 1.85 (0.15) | 2.33 (0.83) | 1.86 (0.02) | 1.84 (0.06) |
| Overall mean | 2.39 (0.81) | 2.37 (0.92) | 1.94 (0.56) | 1.92 (0.11) |

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