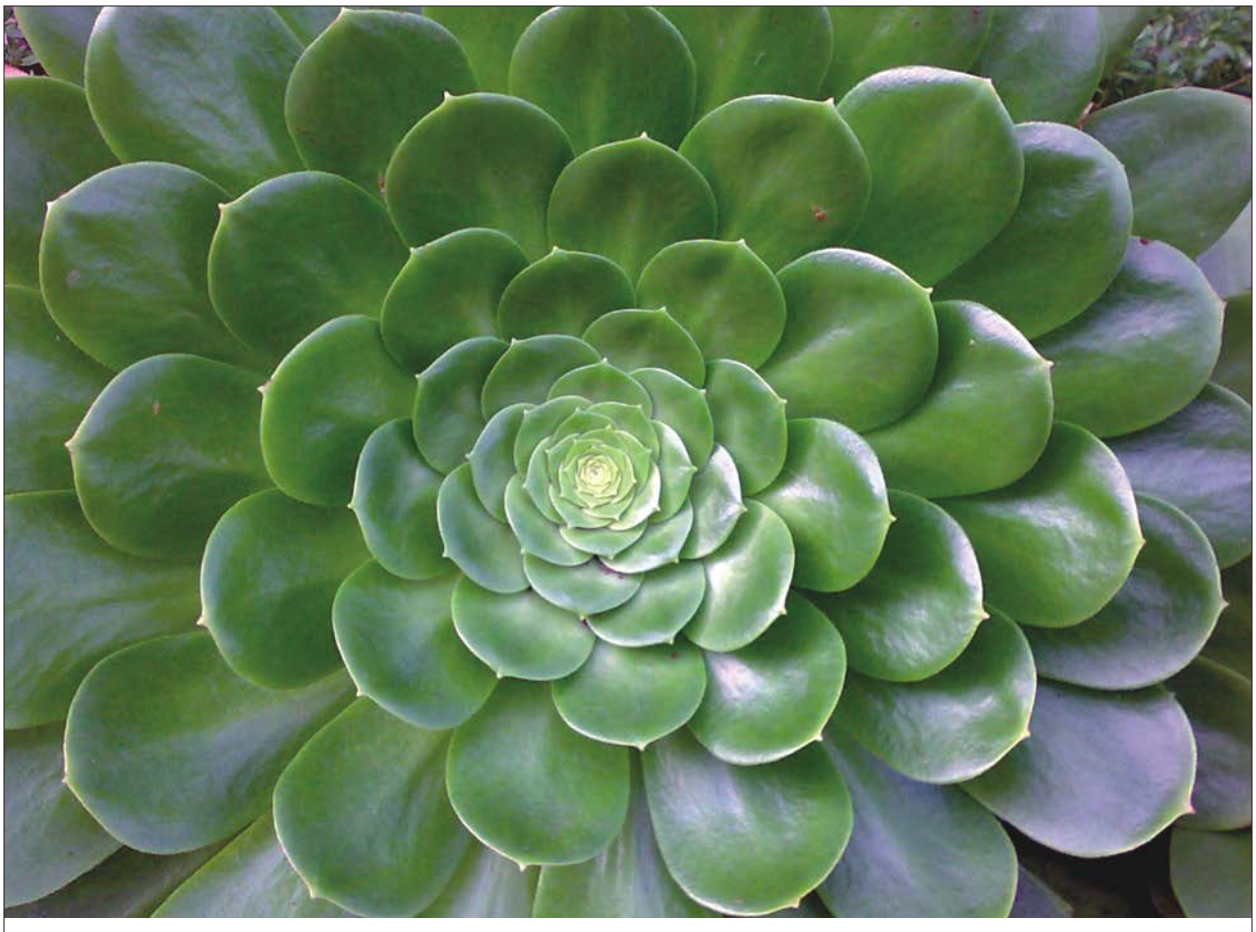

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A DNA Isolation Protocol Suitable for RAPD Analysis from Fresh or Herbarium-Stored Leaves of a Historic *Quercus virginiana* L.

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ABSTRACT

This study described a fast, high-quality yielding protocol for the extraction of genomic DNA from either fresh or herbarium-stored plant tissue of an historic *Quercus virginiana* called the "Emancipation Oak". The protocol was based on the combination of two existing methods-CTAB (hexadecyltrimethylammonium bromide) and DNAzol. The combined DNAzol®-CTAB (C-DC) procedure avoids the traditional phenol: chloroform extraction which was needed to remove polysaccharides and proteins that were normally found in DNA isolated from plants. The C-DC protocol was compared to two standard methodologies for the isolation of intact DNA from either fresh or herbarium-stored plant tissue. The C-DC methodology yields final DNA (from either fresh or herbarium-stored samples) that can be used for RAPD analysis without the need to treat with RNAase. Differences in DNA yields among the three protocols with either fresh or herbarium-stored tissue were analyzed via an ANOVA. The isolated C-DC DNA was free of polysaccharides, polyphenols, and other major contaminants as judged by A260/280 ratio, 0.8% agarose gel electrophoresis and RAPD suitability. This new C-DC protocol will be of great help to study the genetic diversity of the "Emancipation Oak",as well as other species of *Quercus*.

Key words: *Quercus virginiana*, DNA isolation, RAPD analysis, herbarium-stored samples, Hexadecyltrimethyl-ammonium bromide (CTAB)

INTRODUCTION

Over the past forty years, the ability to manipulate DNA has helped to accompany the 'genomics' era of modern molecular investigation. The popularity of genomics has lead to a wide spread need to isolate DNA from a number of organisms to be used in 'downstream' PCR-based amplification schemes. DNA is required for genetic mapping, cultivar identification, taxonomy, phylogenetic studies of animals and medicinal plants, as well as clinical disease studies (Hu and Brady, 2003; Reiter *et al*., 1992; Fu *et al*., 2003; Lynch and Milligan, 1994; Lander *et al*., 1987; Yasmin *et al*., 2006; Champasri *et al*., 2008; Williams *et al*., 1990; Cler *et al*., 2006). DNA and PCR based applications have become popular because they have a number of advantages over cultivar identification tests, in that these applications are unaffected by environmental factors or the developmental stage of the plant. For example, the localization of genetic markers associated with plant disease-resistance has become a major application for DNA genomics (Jena and Mackill, 2008).

Two of the most popular DNA techniques are random amplified polymorphic DNA (RAPD) and Amplified Fragment Length Polymorphism (AFLP) analysis (Xu *et al*., 2004; Vos *et al*., 1995). Both of these techniques require highly purified, intact DNA which requires special treatment of the starting material (i.e., avoiding inadequate storage of the starting material because it decreases the possibility of intact DNA). A growing area of interest for applying DNA genomics has been with systematic studies using stored herbarium specimens. A common shortfall of herbarium specimens has been isolating intact DNA that can be used in molecular applications. As is the case with most plant materials, herbarium-stored sample DNA is contaminated by high concentrations of tannins, phenolics and polysaccharides (Do and Adams, 1991; Lister *et al*., 2008). All of these compounds must be removed if the DNA is to be used in a PCR-based application. A number of DNA isolation protocols have been optimized and used in various combinations to isolate quality DNA from plants for analyses (Khanuja *et al*., 1999; Shah *et al*., 2000; Warude *et al*., 2003; Sarwat *et al*., 2006; Deshmukh *et al*., 2007). Most plant DNA isolation techniques were designed to remove tannins via the incorporation of soluble Polyethylene Glycol (PEG), guanidine thiocyanate, phenol and/or β-mercarptoethanol in the extraction buffers. Polysaccharides and soluble phenolics were removed by using hexadecyltrimethylammonium bromide (CTAB) (Porebski *et al*., 1997; Tel-Zur *et al*., 1999; Varma *et al*., 2007). If the polysaccharides are not removed they make the DNA sample viscous, glue-like and for downstream applications, they inhibit the Taq polymerase. Compounds such as phenol covalently bind to DNA, making DNA brownish, indigestible and decreasing poor yield because the DNA seems to precipitate with the phenolics compounds. Tannins and phenolics are examples of the types of secondary compounds that are produced in large quantities when processing herbarium-stored samples. The presence of these secondary metabolites has been documented to interfere with further DNA analysis (Cubero *et al*., 1999; Warude *et al*., 2003).

This study presented a new DNA Combined DNAzol-CTAB protocol (C-DC) which was optimized to isolate DNA from either fresh or herbarium-stored leaf material. DNA from fresh and herbarium-stored leaves collected from a historic *Quercus virginiana* (Mill.) known as the "Emancipation Oak" was isolated using C-DC protocol and analyzed by Random Amplification of Polymorphic DNA (RAPDs). The C-DC protocol was rapid, avoided the use of phenol or RNAase and resulted in quality DNA (from either fresh or herbarium-stored) that can be used directly in RAPD or PCR-based analyzes. This study reports, the comparisons of the C-DC protocol to two traditional DNA isolation procedures. The methods were evaluated and compared as to their yields and DNA quality.

MATERIALS AND METHODS

Plant material: The work was conducted by the Group for Microgravity and Environmental Biology at Norfolk State University, Norfolk, VA. Fresh leaves were collected, surface sterilized and stored in -20°C. Herbarium-stored leaf samples were pressed according to USDA 1971 Bulletin 348 and stored in an air tight container. For comparison studies, DNA was isolated according to the method of Porebski *et al*. (1997) using hexadecyltrimethylammonium bromide (CTAB) or by using DNAzol as described by Chomczynski *et al*. (1997).

Reagents and chemicals: Tris-EDTA pH 8.0, 100% Ethanol, 75% Ethanol (cold), Chloroform Plant DNAzol® Reagent (Invitrogen), β-mercaptoethanol, Hexadecyltrimethylammonium bromide (Sigma) (CTAB).

CTAB extraction buffer: The 2.0% (w/v) CTAB, 1.4 M NaCl, 100 mM Tris-HCl, 20 mM EDTA pH 8.0, 0.1% (v/v) β-mercaptoethanol.

DZE solution: The 1 volume of Plant DNAzol with 0.75 volume of 100% ethanol.

Equipment: Microcentrifuge (able to achieve 12,000 g), 2 mL Eppendorf tubes, Liquid Nitrogen, Mortar and Pestle, 1 mL pipette with tips, Vortex, Water bath, UV Spectrophotometer.

Combined C-DC methodology: The leaf material was surface-sterilized in 0.01% (v/v) detergent, with 1% Clorox solution as described by Okpodu (2001a).

Critical step: Fresh tissue was not allowed to warm or become "wet". Allowing the samples to become warm activated proteases and the quality of the final DNA was compromised. It was discovered that a higher yield of DNA resulted if the CTAB extraction buffer was added directly to the mortar and then transfer to the Eppendorf tube. For herbarium dried samples, the mid-vein was not removed from the leaves and the samples were directly homogenized in the mortar in liquid nitrogen.

CTAB extraction buffer (400 μL) was added and the sample was incubated at 65°C for 30 min.

Critical step: The protocol can be scaled-up, but a 1:4 ratio must be maintained (e.g., 0.1 g of starting material for every 0.4 mL of CTAB extraction media). The incubation time can be reduced to 15 min for fresh starting material.

Troubleshooting: The β-mercaptoethanol was added to the CTAB just before the buffer was to be added to the sample.

Cool the sample to room temperature for 5 min. Then add 400 μL DNAzol®; mix by inversion to form an emulsion.

Troubleshooting: If an orbital shaker is unavailable, the sample can be agitated by hand.

Chloroform (400 μL) was added and the sample was vortexed vigorously and incubated for 5 min on a shaker (at room temperature) and then centrifuged at 12,000 g for 10 min (at room temperature).

A 1 mL pipette was used to remove the supernatant (top layer only) to a fresh 2 mL Eppendorf Microcentrifuge tube.

Critical step: Do not remove any of the bottom layer of the solution. Depending on the chlorophyll content of the starting material the bottom level will appear "green". The green color is a combination of the pigments in the sample and the DNAzol® Reagent.

Troubleshooting: The procedure can be stopped at this point. Samples are stable for 1 week at room temperature or for at least 6 months at -20°C.

100% Ethanol (300 μL) was added to the supernatant and mixed by inversion.

The sample was incubated at room temperature for 5 min; centrifuged (5,000 g at room temperature) for 5 min. The supernatant was decanted and discarded and the pellet was saved.

To the pellet, 400 μL of DZE was added; vortexed and incubated for 5 min at room temperature. The sample was centrifuged for 4 min at 5,000 g.

The supernatant was decanted and the pellet was washed vigorously with 400 μL of cold 75% ethanol; Centrifuge for 4 min at 5000 g.

The supernatant was decanted and the Eppendorf tube was inverted on a Kim wipe for 1 min. The sample was allowed to air-dry pellet for an additional 5 min.

The pellet was re-suspended in 100 μL of TE pH 8.0. The DNA concentrations were measured using a UV-Spectrophotometer.

RAPD analysis: RAPD analysis was performed in a 25 μL volume of reaction mixture containing 1 X Taq Polymerase Buffer (with 25 mM $MgCl_2$), 0.6 units of Taq DNA Polymerase (Amersham), 5 mM dNTPs, 10 mM of random decamer primer and 15 ng of total genomic DNA. Amplifications were carried out using a DNA thermal cycler with the following parameters: One cycle at 94°C for 2 min, 36°C for 2 min and extension at 72°C for 2 min; 29 cycles of denaturation at 94°C for 1 min, primer annealing at 42°C for 1 min and extension at 72°C for 1 min and final extension at 72°C for 10 min. The amplified products were size fractionated on 0.8% agarose gel (1X TBE) and visualized under UV light after ethidium bromide staining. The amplification products were by arbitrary primer OPA-6 a decamer primer, (5-'GCTCCCTGAC-3'), from Operon Technologies, USA).

Statistical analysis: Differences between means were analyzed by an Analysis of Variance (ANOVA). The ANOVA was performed using a web-based free statistics calculator created for scientists, researchers and students (Soper, 2009). Values presented were the means plus and minus the Standard Error of the Means (SEM) (****, $p<0.05$). The software compares three different protocols for DNA extraction and two types of samples (fresh and herbarium-stored). A Tukey Honestly Significant Difference (HSD) was used to compare all pairs of means following the one way ANOVA. A box and whisker plot was constructed using the method of Hunt (1996).

RESULTS AND DISCUSSION

Qualitative differences of the three DNA methods: Isolation of DNA from leaf tissue can be hard due to high levels of polysaccharides and polyphenols (Do and Adams, 1991). Compared to the other methods, the C-DC method yields predominant high molecular weight DNA (Fig. 1, lanes 11-13). Although both the CTAB and DNAzol methods yield DNA, the samples have low molecular weight contaminants, sheered-DNA regardless of the starting material (i.e., fresh or herbarium-stored) when examined on a 0.8% agarose gel (Fig. 1, lanes 1-7).

Length of procedure and final purity index: Table 1 is a summary of all methods used to evaluate both DNA quantity and quality. The DNAzol purity index for both fresh and herbarium samples were very poor (1.39±0.09 and 1.04±0.075, respectively). The DNAzol method was fast (1.5 h); however, steps to remove RNA contamination by the addition of ribonuclease A lengthen the procedure to a total of three hours. These findings are in agreement with those of Guo *et al.* (2005) who also found that although the DNAzol procedure was rapid, modifications required in order to obtain DNA of satisfactory yield and purity lengthened the procedure.

The CTAB method took the most time; however, when compared to the DNAzol method on a cost comparison analysis as completed by Chen *et al.* (2010), both the DNAzol and CTAB methods cost $3.30 per sample. The biggest difference between the CTAB protocol and the DNAzol and C-DC

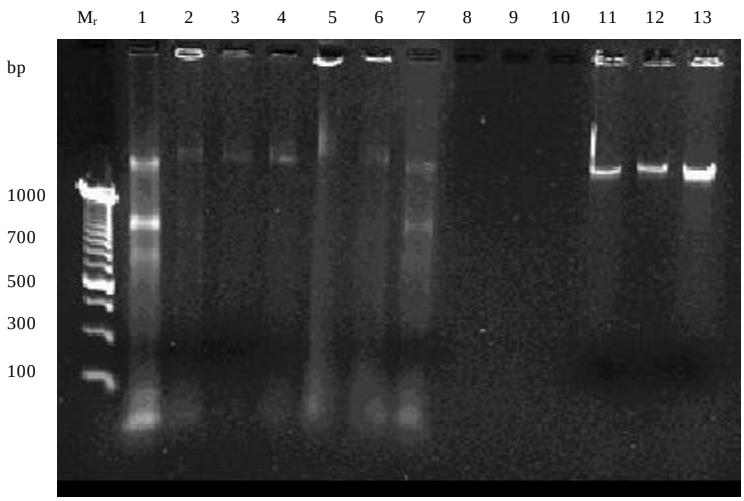

Fig. 1: Electrophoresis of extracted DNA on a 0.8% agarose gel. Lane 1, is (10 μg) DNA isolated with the CTAB method from fresh leaves; Lanes 2-4 is (10 μg) DNA isolated using CTAB method with herbarium-stored leaves; Lane 5-6 DNA (10 μg) isolated from herbarium-stored leaves using DNAzol®; Lane 7, is DNA isolated from fresh leaves using the DNAzol® protocol. Lanes 8-10 are open lanes. Lanes 11-13, DNA isolated using the C-DC method with varying concentration of DNA (2.5 and 10 μg, respectively). Lane 11 has DNA isolated from herbarium (1.0 μg) stored leaves and lanes 12 and 13 is DNA isolated from fresh leaves. M_r is 5 μL of 100 bp ladder (Invitrogen)

Table 1: Spectrophotometric analysis of DNA extracts using C-DC, CTAB and DNAzol® from fresh and herbarium-stored leaves and the time associated to analyzed 12 samples. All samples were re-suspended in 100 μL of TE pH 8.0

Protocol	Time (h)	% yield (μg g^{-1})	A_{260nm}/A_{280nm}
Herbarium-stored samples			
C-DC	1.5	12.20±2.40	1.40±0.28
CTAB	8.0	14.50±3.98	2.20±0.14
DNAzol®	1.5	7.10±1.40	1.39±0.09
Fresh sample			
C-DC	1.5	36.30±1.80	1.95±0.0078
CTAB	8.0	15.59±1.20	1.87±0.015
DNAzol®	1.5	34.60±1.70	1.04±0.075

The results are the Averages±Standard error of the mean from three different extractions

methods was the CTAB used chloroform: isoamy-alcohol treatments to remove protein and other contaminants from the DNA sample. In general CTAB resulted in genomic DNA yields comparable to the C-DC protocol. According to the purity index, the CTAB method yield 2.20±0.14 and 1.87±0.015 for both herbarium-stored and fresh samples, respectively. Although the CTAB purity index was comparable to the purity index for the C-DC method for both fresh and herbarium stored leaves (Table 1), the CTAB method required 8 hours to analyze twelve samples compared to the C-DC method which took 1.5 h for twelve samples. DNA isolation using the CTAB method did not always yield DNA that either resulted in reproducible results or could be used immediately in

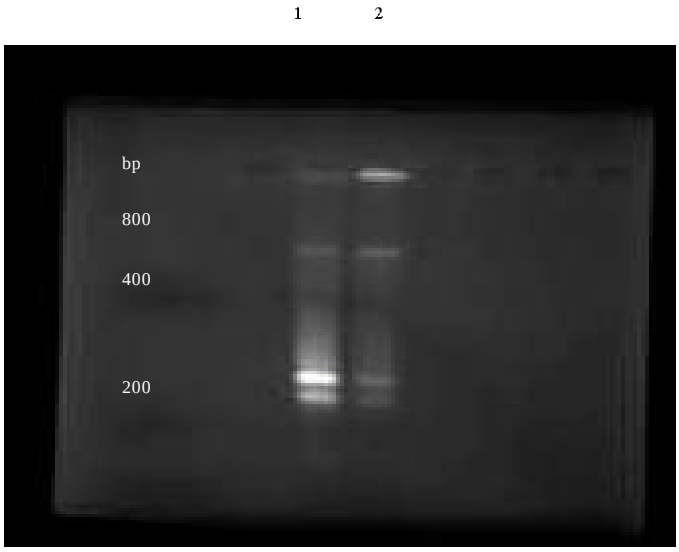

Fig. 2: RAPD analysis of DNA isolated from either fresh (lane 1) or herbarium specimen leaves (lane 2) using C-DC. RAPD analysis was performed on two separate leave samples. The DNA in lane 1 was isolated from leaves that had been collected but not prepared for herbarium storage. The DNA in lane 2 was from leaves that had been pressed and dried. The amplification products were by arbitrary primer OPA-6 a decamer primer (5-'GCTCCCTGAC-3'), from Operon Technologies, USA). A total of three bands were amplified in lane 1 and four bands in lane 2. The base pair sizes of the DNA have been indicated on the gel

downstream PCR-based applications without additional steps to clean the sample of RNA contamination (data not shown). However, the DNA isolated via the C-DC protocol was quick (1.5 h), inexpensive (yield/time) and could be used for RAPD analysis (Fig. 2) without the need to treat with RNAase. Other methods like CD-C that involve modifications of the CTAB protocol have also proven successful in yielding high molecular weight DNA that is suitable for PCR applications (Srivastava *et al*., 2010; Sahasrabudhe and Deodhar, 2010).

Quantitative differences for three DNA methods for fresh and herbarium-stored samples: For DNA quantification, we used spectrophotometer and visualization of band intensity of DNA samples separated on an agarose gels. DNA quality was estimated by agarose gel electrophoresis and used for RAPDs (Fig. 3). Among the three protocols examined, the combined CTAB (C-DC) protocol proved efficacious for DNA isolation, especially for fresh material. The average yield of DNA following the C-DC protocol was 12.2±2.4 $\mu g.g^{-1}$ dry weight of leaf material and 36.30±1.80 $\mu g.g^{-1}$ fresh leaf material (Table 1). The average yield C-DC gave the higher DNA concentration under both conditions as compared with DNAzol ($p<0.05$). Chen *et al*. (2010) also found that methods utilizing CTAB yield significantly higher quantities of DNA when compared with those obtained using DNAzol.

We used a simple graphic method called the "box plot" (also called a schematic plot or box-and-whiskers plot) to summarize and interpret the data. Statisticians recognized the box and whisker

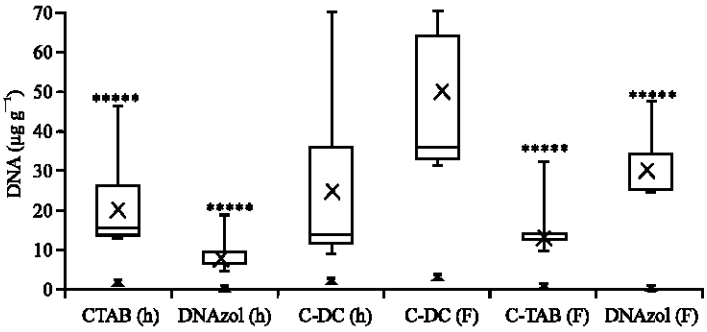

Fig. 3: A Box-and-Whisker Plot comparing the DNA concentration of three DNA isolation methods using either herbarium or fresh leaf materials. The boxes in the plots represent the 25th and 75th percentiles. The lines in the boxes represent the 50th percentile. The "X" indicates the mean of the sample set (n = 9). The whiskers represent the highest and lowest value in the data set. DNA isolation was significantly lower in the DNAzol method, both for herbarium-stored and fresh leaf materials as compared to both the CTAB and C-DC methods. Means that were significantly different ($p<0.05$) from the C-DC mean of the respective starting material (i.e., fresh or herbarium-stored) are indicated by **** (Tukey's HSD test)

plot as one of a diverse family of statistical techniques that can be used to visually identify patterns that may otherwise be hidden in a data set. The box and whisker plot (Fig. 3) compares the three protocols for the two different samples (i.e., fresh or herbarium-stored). DNA isolation was significantly lower in the DNAzol method regardless of the starting material (i.e., fresh or herbarium-stored) when compared to both the CTAB and C-DC methods. In Fig. 3, means that were significantly different were determined by the Tukey's HSD post ANOVA analysis and were indicated by Tukey (1991). From the Tukey's post-hoc test we found that there were significant differences between the means of the C-DC method and DNAzol procedure for both fresh and herbarium-stored. In comparison to the two standard protocols (i.e., DNAzol and CTAB individually), the C-DC protocol yield a 70-100 fold increase in the amount of total genomic DNA isolated (Table 1) and no RNA is evident when the final products are separated on agarose gels (Fig. 2, lanes 11-13).

CONCLUSION

In this study, we described the development of a new protocol for the extraction of genomic DNA from fresh plant tissue and herbarium-stored samples based upon a combination of two existing protocols - CTAB (hexadecyltrimethylammonium bromide) and DNAzol®. This procedure, combined DNAzol®-CTAB (C-DC) protocol, avoids the traditional phenol: chloroform extraction to remove polysaccharides and proteins that are normally found in DNA isolated from plant materials. As was described by Lister *et al.* (2008) "herbarium and other historic collections of plants, fungi and animals have been recognized as an important genetic resource useful, for example, in resolving taxonomic questions, inferring evolutionary history and determining historical distributions of populations." Present research grew from an interest in getting minority students in the United States to be interested in various aspects of plant biology (Okpodu, 2001b). The project, "The Emancipation Oak-interdisciplinary living laboratory" was presented in 2003 at the Heritage and

History National Conference of the National Association of African American Studies in Houston, Texas (Okpodu, 2003). This project describes an interdisciplinary laboratory project that integrated history with current biotechnology techniques to address a plant systematic question centered on understanding the identity of a historic oak tree called the Emancipation Oak. Project O.A.K. (Opportunities Alliance networK) used a bilateral approach to establish an undergraduate training and cellular biology research center that incorporated research with teaching. In addition to the research project on DNA isolation and RAPD analysis, students were taught how to identify various plant pathogens that affect oak species.

Oak trees in the United States are highly susceptible to Oak wilt, caused by *Ceratocystis fagacearum* (Wilson, 2005). In 2005, Wilson estimated that *C. fagacearum* caused well over $1 billion (US dollars) in economic damage to trees in Texas alone. Although the "Emancipation Oak" was not affected by *C. fagacearum*, a different fungus, *Taphrina caerulesens,* was identify and isolate from the leaves of the tree (Okpodu, 2001a).

The "Emancipation Oak" tree is located in the city of Hampton, Virginia. It has been recognized as both a State and National Historic landmark (Okpodu, 2001b). It is the site where President Lincoln signed the Emancipation Proclamation to end slavery in the United States. According to a local historical society called The Contraband Slave Historic Society which is an advocacy group for documenting the contribution of African Americans at Fort Monroe, Virginia there are several trees in the Hampton Roads Area that were somatically cloned from "the Emancipation Oak (Okpodu, 2003). Therefore, this research uses an ethno-botanical approach to integrate biological principles to teach undergraduate students and to resolve a historic taxonomical hypothesis that the Emancipation Oak was the parent plant to a local tree that lacked any formal information only local folklore. In the course of the work, it was discovered that several trees that are historical significance in the Hampton Roads Area may be genetically related to the Emancipation Oak and these trees had been slated to be removed. In addition, the Group for Microgravity and Environmental Biology (GMEB) was approached by a local advocacy group who asked us if a genomic approach could be used to address this question specifically.

It was decided that the random amplified polymorphic DNA analysis would be used to address the question of genetic relationship among these historically significant trees. Plant materials were obtained from either fresh leaves or herbarium specimens. RAPD technique was easier than AFLP because it many more samples could be processed in a short period of time; however, RAPD analysis required that the template DNA was clean and not contaminated with RNA.

The application of molecular tools to analyze DNA from herbarium-stored plant material has been described and used from a wide range of museum and herbarium specimen (Lister *et al*., 2008; Cubero *et al*., 1999). Using the method described by Porebski *et al*. (1997) or Chomczynski *et al*. (1997) with the Emancipation leaf samples resulted in contamination from polysaccharides, phenolic compounds and RNA. With the DNAzol method, samples turned dark brown after three days of storage at 4°C and the samples did not always amplify in the RAPD assays.

The classic CTAB protocol presented certain limitations: (1) CTAB was time consuming and did not allow flexibility when working with lots of samples; (2) CTAB required a phenol-chloroform extraction in order to have the sample suitable for RAPD or PCR analysis (Ye *et al*., 1996; Doyle and Doyle, 1987; Stewart and Via, 1993) and (3) a 3-5 fold over overestimation of the final yield of DNA because of RNA contamination and other secondary plant metabolites (Csaikli *et al*., 1998).

The commercially available DNAzol®protocol was relatively quick (~1.5 h) but resulted in more RNA than DNA from woody tissue than the C-DC combined protocol. The C-DC protocol was used with as many as twenty-four samples per isolation. Considering that the method took less than two hours to complete, the procedure was easily scaled-up to extract more than 50 samples in one day. The C-DC method for isolating genomic DNA for further analysis was ideal for working with students. The phenol: chloroform step was eliminated and it allowed for DNA to be isolated without additional enzymatic treatment.

The C-DC protocol was very powerful in helping to address the original goal which has been to complete genetic studies on the relatedness of several trees that are believed to be derived from the "Emancipation Oak." Having DNA that can be amplified from either fresh or stored herbarium samples now allows the GMEB to fully address the question for both current and historic population of trees of the Emancipation Oak that may exist.

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