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IN SITU PRESERVATION OF DNA IN PLANT SPECIMENS

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Summary

Twenty-seven treatments of plant specimens were examined to determine their effects on the quantity and quality of DNA obtained from spinach leaves. Treatments included various drying methods, cold storage, and chemical preservatives. Good yields of quality genomic DNA (30–50 kbp) were obtained from fresh, frozen, and dried leaves, but none of the chemical preservatives yielded high molecular weight DNA, even after only three to seven days of storage. The application of the hot CTAB procedure to fresh and dried leaves of *Ginkgo biloba, Juniperus ashei, J. virginiana, Magnolia grandiflora, Phoradendron tomentosum, Pinus ponderosa, Quercus virginiana* and *Thuja orientalis,* species that possess considerable tannins, yielded high molecular weight DNA from all the fresh leaves with some degradation. The dried leaves of *Juniperus ashei, Quercus virginiana* and *Magnolia grandiflora* yielded only small amounts of high molecular weight DNA with the hot CTAB extraction procedure.

Introduction

The extraction of high molecular weight DNA from plant materials has been detailed in the works of Bendich et al. (1980), Murray and Thompson (1980), Fluhr and Edelman (1981), Rivin et al. (1982), Zimmer and Newton (1982), Palmer (1982, 1986), Palmer and Zamir (1982), Palmer et al. (1985), and Rogers and Bendich (1985) to name but a few.

Arrighi et al. (1968) examined DNA from liver tissue and *in vitro* cells of the Chinese hamster stored for six to 66 days in 95% ethanol; absolute methanol; 1 methanol : 9 ethanol; 1 methanol : 1 ethanol; 3 methanol : 1 glacial acetic acid; 2-propanol; acetone; and 10% formalin. Physical properties (UV absorbances at 260, 230, 280 nm), buoyant density, melting curves, and hyperchromicity of the extracted DNAs were determined. They concluded that "Recovery of 'normal' DNA was excellent with most fixatives, but not when formalin was used." They recommended 2-propanol or ethanol as fixatives. However, since no electrophoresis gels were run, DNA sizes are unknown. Rake (1972) noted that the work of Arrighi et al. (1968) was merely suggestive of DNA preservation and that it did not prove that "DNA fixed in isopropanol was in fact suitable for DNA-DNA reassociation techniques." Preservation of *E. coli* in 95% isopropanol for five to 14 days (Rake, 1972) resulted in DNA that was shown to be suitable for reassociation studies. However, because the extracted DNA was sheared to about 500 bp before reassociation tests and no electrophoresis sizing was performed on the extracted DNA, one is still left with uncertainty as to the size of the extracted DNA.

Only a few papers have been published dealing with the preservation/degradation of DNA in plant specimens. Rogers and Bendich (1985) obtained DNA with a maximum length of 20 to 30 kbp with average lengths of 0.1 to 1.0 kbp from herbarium vouchers ranging in age of 20 to 95 years old. DNA from *Juniperus osteosperma* seeds from pack rat middens (3500; 27,000; and greater than 45,000 yr bp) yielded DNA with maximum lengths of 10, 10, and 10 kbp and average lengths of 7, 5, and 3 kbp, respectively.

More recently, Doyle and Dickson (1987) reported on efforts to preserve *Solanum* leaves using formalin-acetic acid-ethanol (FAA), Carnoy's solution (ethanol : acetic acid, 3:1), 70% ethanol, chloroform : ethanol (4:3), brine solution (10% NaCl), and drying at 42°C. Essentially none of the chemical treatments preserved DNA for a week, except the chloroform :

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ethanol. However, the DNA was lost from it after three weeks. Doyle and Dickson (1987) did get good DNA from the dried leaves. The brine solution developed fungal contamination which likely lysed the cell membranes.

Due to the importance of preserving tropical plant materials for subsequent DNA extraction and analyses, we have investigated additional methods of preservation and attempted to extend the hot CTAB procedure to more difficult species such as oak, juniper, and magnolia. The purpose of this paper is to report on the effects of various temperatures and chemicals on the preservation of DNA *in situ.*

Preparation of Plant Material

Leaves from fresh spinach (*Spinacia oleracea* L.) were purchased locally. *Juniperus ashei* Buch., *J. virginiana* L., *Phoradendron tomentosum* (DC.) Gray, and *Quercus virginiana* Mill. were collected from native trees near the Baylor campus. *Pinus ponderosa* Dougl. ex P. Lawson was obtained from Dryden, Washington (Adams and Edmunds, 1989). *Ginkgo biloba* L., *Magnolia grandiflora* L., and *Thuja orientalis* L. were collected from cultivated trees on the Baylor campus.

Approximately 0.5 g (FW) of leaf was used for each of the treatments (see Table 1). Leaf drying (treatments 4, 5) was done in a conventional plant press dryer at 42°C for 24–48 h. Desiccation was performed in a desiccator over Drierite (anhydrous $CaSO_4$). For treatment 7, fresh spinach was placed on the lab bench and dried at room temperature. Microwave drying was for three minutes at a setting of high (700 watts). The various chemical treatments are listed in Table 1. In each chemical treatment, the leaf material was added to a vial of the chemical solution, capped, and stored at 37°C for the time indicated in Table 1.

DNA Extraction and Analyses

The hot CTAB procedure (Doyle and Dickson, 1987; Doyle and Doyle, 1987) was used with minor changes as noted herein. Fresh spinach leaf material (0.5 g FW) was weighed and placed in a preheated (60°C) mortar and pestle with preheated (60°C) 3.5 ml 2× CTAB buffer (2× CTAB = 2% (w/v) hexadecyltrimethylammonium bromide (Sigma H5882), 1.4 M NaCl, 100 mM Tris-HCl pH 8.0, 20 mM EDTA, 0.2% (v/v) 2-mercaptoethanol (Sigma M3148)). The leaves were ground and the grindate transferred to a polyallomer centrifuge tube via a wide-tipped Pasteur pipet. The mortar and pestle were washed with 0.5 ml of 2× CTAB buffer and the wash added to the centrifuge tube. The centrifuge tube was then incubated for 30–45 minutes at 60°C. After incubation, an equal volume of 24:1 chloroform : isoamyl alcohol was added and mixed by shaking gently, then centrifuged ten minutes at 8500 × g. The aqueous layer was removed using a wide-tipped Pasteur pipet, and transferred to a clean centrifuge tube to which two-thirds volume cold (−15°C) isopropanol was added and mixed. The DNA was pelleted by centrifugation for one minute at 1000 × g. Longer, harder spins were used as necessary to get the DNA to stick to the wall of the tube. The DNA pellet was washed with buffer (8 ml, 76% EtOH, 10 mM NH_4OAc) at room temperature for from 20 minutes to overnight. The DNA was then pelleted by high speed centrifugation for 10 minutes at 8500 × g and the wash buffer carefully decanted. The DNA pellet was air dried for 30 minutes then redissolved in 500 μl of resuspension buffer (10 mM NH_4OAc, 0.25 mM EDTA). RNase A (Sigma R5000) was added to a final concentration of 10 μg/ml and incubated 30 minutes at 37°C as per manufacturer's instructions. Two (2.0) volumes (1 ml) of dI water was added along with 848 μl of 6.92 M NH_4OAc (2.5 M final conc.) and the DNA was then precipitated by the addition of 2.5 volumes (5.87 ml) of cold 95% ethanol and centrifugation for 10 minutes at 8500 × g. The DNA pellet was air dried at room temperature overnight and the DNA resuspended in 0.5 ml of 1× TE (1 mM Tris-HCl, 0.1 mM EDTA, pH 7.2) buffer.

For gel electrophoresis, DNA was mixed in various concentrations with 1× TBE (89

Table 1. DNA sizes from spinach versus method of storage for spinach leaves. NT = not tested further. Sizes are the ranges, given in kbp.

Treatment	Sampled after: 24 h	1 mo	2 mo	5 mo
1. Fresh	23–49	23–49	23–49	23–49
2. Frozen at −20°C	23–49	23–49	23–49	23–49
3. Kept refrigerated at 4°C	23–49	23–49	23–49	23–49
4. Dried 42°C, stored −15°C	23–49	23–49	23–49	23–49
5. Dried 42°C, stored 22°C	23–49	23–49	23–49	23–49
6. Desiccated, stored 22°C	23–49	23–49	23–49	7–0.5
7. Dried 22°C, stored 22°C	23–49	23–49	23–49	7–0.5
8. Blanched in boiling water	<0.5	NT	NT	NT
9. Microwaved, 3 min	7–0.5	NT	NT	NT
10. 1:3 acetic acid/ethanol	<0.5	NT	NT	NT
11. 95% ethanol	<0.5	NT	NT	NT
12. Glycerol	<0.5	NT	NT	NT
Sampled after: 3 days				
13. Tap water . . . not sampled due to fungal growth and rotting				
14. 3% Na azide	3.5–0.5			
15. Perfix preservative	<0.5			
16. Pentachlorophenol/chloroform (1.5/98.5 v/v)	<0.5			
17. Paraformaldehyde, 10%	<0.5			
18. 1% guanidine thiocyanate	3.5–0.5			
Sampled after: 7 days				
19. NaCl, 25% soln.	3.5–0.5			
20. Clorox, 5% soln.	<0.5			
21. Methanol/chloroform/propionic acid 1:1:1	<0.5			
22. Glutaraldehyde, 8%	<0.5			
23. Formaldehyde, 7.4%	<0.5			
24. Trichloroacetic acid, 10%	<0.5			
25. Glutaraldehyde, 2%; 0.05 M Na cacodylate, pH 6.8	<0.5			
26. EDTA, 10 mM	<0.5			
	Sampled after: 14 days	1 month		
27. EDTA, 0.5 M	7–0.5	<0.5		

mM Tris base, 89 mM boric acid, 2 mM EDTA, pH 8.0) and one-fifth volume of loading buffer [15% Ficoll w/v (Sigma F 2637) or 40% sucrose w/v, 0.05% bromphenol blue, and 0.10 M EDTA in 1× TBE]. Ten µl of this mixture was loaded onto a 0.6% agarose gel (Sigma A6013), submerged in running buffer (1× TBE, 0.5 µg/ml ethidium bromide) and electrophoresed for 30 minutes at 100 V (10 V/cm), using a Biorad Mini-Sub cell and model 100/200 power supply. Concentrations of the extracted DNA's were estimated by comparison with unrestricted lambda DNA (Sigma D0144) loaded in various known concentrations. DNA sizing and degradation were determined by electrophoresis on a 0.3% agarose gel, run for 60 minutes at 100 V (10 V/cm) and otherwise as above. Size markers were: coliphage T5 DNA (103 kb, Sigma D8010), unrestricted lambda (48.5 kb) (Sigma D0144), and *Hind* III digested lambda DNA. Restrictability was checked using *EcoRI*, *Hind* III, *Kpn* I, and *Sal* I (Sigma R2132, R1882, R9506 and R0754, respectively). When restricted plant DNA's were electrophoresed, lambda DNA restricted with *Hind* III (Sigma

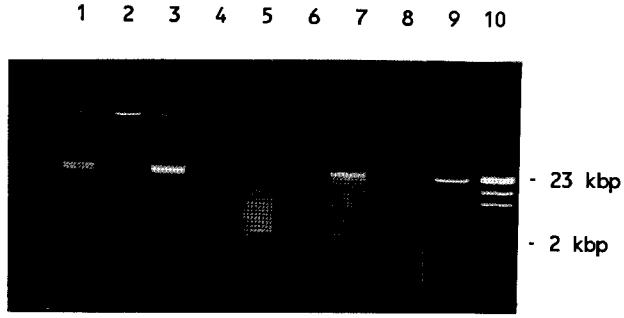

Fig. 1. Comparisons of DNA from fresh versus air dried (42°C, 24–36 h then stored at 22°C for 3½ months) leaves of *Juniperus ashei* (fresh, lane 1; dried, lane 2); *J. virginiana* (fresh, lane 3; dried, lane 4); *Magnolia grandiflora* (fresh, lane 5; dried, lane 6); *Quercus virginiana* (fresh, lane 7; dried, lane 8); lambda DNA (48 kbp, lane 9); lambda DNA restricted with *Hind*III (lane 10).

R1882) was co-run to provide size standards. Gels were photographed under short wave UV light using a Polaroid direct screen camera (DS34).

Results and Discussion

The results from the treatments are shown in Table 1. With the exception of the 0.5 M EDTA, none of the chemical treatments was successful in preserving the DNA in spinach leaves, not even in the initial sample after 24 h. The failure of the chemical treatments to preserve DNA agrees with Doyle and Dickson (1987), but is in contrast to Arrighi et al. (1968) and Rake (1972). But as noted previously, neither of these studies examined DNA size nor restrictability. Interestingly, Scott and Timmis (1984) "fixed" spinach roots for 1–2 h in 0.5% formaldehyde in 0.01 M HEPES before extracting DNA. The DNA appeared to be smaller after fixing but still with restriction bands of 2–8 kbp. They also note that cpDNA was equally restrictable from unfixed chloroplasts and those fixed in 4% formaldehyde. It is hypothesized that the longer exposure to formaldehyde (one week by Doyle and Dickson, 1987; and this paper) is more disruptive than the 1–2 h exposure (and lower concentration) used by Scott and Timmis (1984).

In contrast to the chemical treatments, the DNA was fairly well preserved from air dried and desiccated leaves although there appears to be some loss of DNA in the dried leaves in the two month samples. Of course, freezing the leaves gave good, high molecular weight DNA but storage and transport while frozen in the tropics is difficult and sometimes not possible. The use of the hot (55°–60°C) CTAB extraction buffer prompted us to consider heating the leaves quickly (blanching in boiling water) to denature DNases that degrade the DNA. This apparently did not denature the DNases, because we got no DNA (Table 1) from this treatment. Subsequently, we found out that DNA restriction enzymes have varying resistance to heat denaturation. Many DNases are not denatured until temperatures of over 60°C and up to 100°C are reached (U.S. Biochemical Corp., 1988 Catalog, Cleveland, OH). It appears that the DNA was denatured before the DNases have been denatured. If so, this would explain the failure of the blanching treatment.

Microwave drying was tried as an alternative method of drying. The DNA was totally degraded. Additional experimentation with power levels and time might produce dried specimens that contain good DNA. Further experimentation on microwave drying will be needed.

In summary, only the dried, refrigerated and frozen foliage gave high molecular weight DNA from spinach (an easy species from which to obtain DNA). We have also tried the

hot CTAB extraction methods (Doyle and Doyle, 1987) on several species that are high in tannins and other secondary compounds that interfere with DNA extraction.

The quality of the DNA was examined by restriction enzymes. In each case where DNA of 30–50 kbp size was obtained, it could be restricted by *EcoRI, Hind* III, *Kpn* I and *Sal* I.

The application of the hot CTAB procedure to fresh leaves of *Ginkgo biloba, Juniperus ashei, J. virginiana, Magnolia grandiflora, Phoradendron tomentosum, Pinus ponderosa, Quercus virginiana,* and *Thuja orientalis* yielded high molecular weight DNA. However, comparisons of fresh and air dried (42°C, 24 to 48 h; then stored at 22°C for 3.5 months in a herbarium cabinet) mature leaves of *Juniperus ashei, J. virginiana, Magnolia grandiflora,* and *Quercus virginiana* revealed considerable differences among these species. Both juniper species yielded rather similar results: the yields of DNA from fresh leaves of *J. ashei* and *J. virginiana* were approximately ten times as large as yields from dried (3.5 mo) leaves. Both junipers yielded DNA ranging in sizes from 49 to 103 kbp (Fig. 1).

Fresh leaves of *Magnolia grandiflora* yielded some DNA that was rather degraded (2–20 kbp). Dried magnolia leaves gave almost no DNA. Fresh oak leaves produced yields of DNA comparable to juniper with approximately 90% of the DNA ranging in sizes from 23 to 49 kbp, with the balance ranging down to 2 kbp (Fig. 1).

The variable yields of high molecular weight DNA are likely due to complexing of phenolics/tannins as well as polysaccharides either before or during extraction (Coradin and Giannasi, 1980; Doyle and Dickson, 1987; Doyle and Doyle, 1987; Hattori, Gottlob-McHugh and Johnson, 1987; Locy and Hall, 1978; Murray and Thompson, 1980). Although we did not find a totally satisfactory method for preservation, drying seems to hold the most promise at present. Additional research (in progress) on modifications of the extraction buffer may yield more and higher molecular weight DNA from dried material.

In any case, the recent advances in the technology for the extraction and immobilization of DNA, coupled with the prospect of the loss of significant plant genetic resources throughout the world, warrant a critical examination of the interactions of natural products with DNA during extraction and the development of efficient methods for the preservation of DNA for a few weeks under field conditions until the plant material can be shipped to a facility for DNA extraction.

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