Interim and Archival Preservation of Plant Specimens in Alcohols for DNA Studies

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ABSTRACT

Three plant species, spinach, juniper and broccoli, were stored in different alcohol solutions, and the DNAs were examined to determine changes in the quality and quantity of DNA over time. The smallest changes in the genomic DNA were found in the samples stored in the lower mol wt alcohols (100% methanol and 100% and 95% ethanol) and when proteinase (Pronase E) was used in the extraction procedure. After 11 months of storage in ethanol, excellent yields of high mol wt DNA were obtained, but only when the procedure utilized Pronase E. The use of proteinase was found to be essential to obtain DNA from preserved specimens of spinach and broccoli. This appears to explain the previous reports of failures to obtain DNA from alcohol-preserved specimens. Vacuum infiltration of ethanol resulted in better DNA preservation than passive infiltration of ethanol.

INTRODUCTION

The preservation of specimens is important for numerous reasons, including forensics, pathology, taxonomy and evolutionary studies. There has been considerable interest in the interim preservation of DNA in situ in plant specimens (see Reference 7 for a recent review). Although silica gel preservation is the accepted method for field preservation (1), liquid methods may be useful under certain circumstances. Doyle and Dickson (3) 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-:ethanol. However, the DNA was lost in the chloroform:ethanol after 3 weeks. Doyle and Dickson (3) did get good DNA from the dried leaves.

Pyle and Adams (9) examined the

effects of chemical preservatives on DNA preservation of spinach. They reported that Carnoy's solution, 95% ethanol, glycerol, 3% sodium azide, Perfix preservative (J.T. Baker, Phillipsburg, NJ, USA), pentachlorophenol/chloroform (1.5:98.5), 10% paraformaldehyde, 1% guanidine thiocyanate, 25% brine solution, 5% Chlorox®, methanol:cholorform: propionic acid (1:1:1), 2% and 8% glutaraldehyde, 7.4% formaldehyde, 10% trichloroacetic acid, 0.5 M sodium cacodylate and 10 mM EDTA did not preserve DNA in situ in spinach for seven days at 37°C. The failure to preserve the DNA in spinach in ethanol seems anomalous in view of the successful use of ethanol for preservation of animal tissue (8,12). Isopropanol (95%) has been used to preserve *Escherichia coli* 5 to 14 days (10).

Post et al. (8) obtained high-quality DNA from insect specimens preserved in liquid nitrogen, ethanol (4°C) and dried over silica gel, but they reported degraded DNA from Carnoy's solution, methanol and propanol treatments and no DNA from pinned specimens or formal saline treatments.

In contrast to plant work, proteinase (usually proteinase K) is frequently utilized in the extraction protocols for chemically preserved animal and human tissues (11,12). The use of ethanol to preserve animal tissues likely results in denaturation of protein (and DNases), as well as the precipitation of proteins. Due to the intimate association of nuclear DNA with histones and other proteins, it seemed plausible that precipitated proteins might coagulate around DNA and sequester the DNA from normal extraction procedures. Thus, we decided to expand the work of Pyle and Adams (9) both with and without the use of proteinase (Pronase E). This led to a more detailed examination of the efficacy of other alcohols for the in situ preservation of DNA.

MATERIALS AND METHODS

Preparation of Plant Material

Leaves from fresh spinach (*Spinacia oleracea* L.) and broccoli (*Brassica oleracea* L.) were purchased locally. Juniper (*Juniperus. virginiana* L.) was collected from trees near the Baylor University campus.

Ten leaf disks (10-mm diameter) of spinach (24 mg), 20 broccoli buds (5 mg) each and 20 leaflets of juniper (8 mg) each were stored in 4-dram vials with an excess amount (2 mL) of various alcohols: 100%, 95%, 70%, 50%, 25% ethanol, and 100% each of methanol, ethanol, propanol, 2-butanol, pentanol, hexanol, heptanol, octanol and decanol. For spinach and broccoli, additional vials were stored that were vacuum-infiltrated with 100% ethanol. The vacuum infiltration process consisted of placing the vial in a vacuum chamber, applying a vacuum of 640-mm Hg for 3 min and releasing the vacuum for 3 min, then repeating the process twice (total of 3 infiltrations). Storage of spinach in Carnoy's solution, glycerol, pentachlorophenol/chloroform (1.5: 98.5 vol/vol), 10% paraformaldehyde, 1% guanidine thiocyanate, 25% sodium chloride, 5% Chlorox, methanol/ chloroform/propionic acid (1:1:1), 8% glutaraldehyde, 7.4% formaldehyde, 10% trichloroacetic acid and 0.5 M EDTA follows the previous study (9).

DNA Extraction and Analyses

Plant material was ground in a micro-mortar, and the DNA was extracted by the hot CTAB procedure (3,4), except that DNA was precipitated by the addition of 2 volumes of ethanol (rather than the use of 2/3 volume of isopropanol used in References 3 and 4). Unless otherwise noted (Figures 3 and 4), Pronase E (Sigma Chemical, St. Louis, MO, USA) was added at the initial extraction step and incubated for 30 min at 60°C. Pronase E was used because it is less expensive than proteinase K. DNA was separated on 1.5% agarose gels by electrophoresis (20 min, 100 V, 10 V/cm) with ethidium bromide in the gel and buffer. DNA quantities and qualities were estimated by comparisons with serial dilutions of genomic mouse DNA (D-0144; Sigma Chemical) and λ *Hin*dIII markers. Gels were photographed over shortwave UV light using a Polaroid direct-screen camera (DS34; Cambridge, MA, USA).

RESULTS AND DISCUSSION

As in the previous study of Pyle and Adams (9), the DNA from spinach stored in Carnoy's solution, glycerol, sodium azide, Perfix preservative, pentachlorophenol/chloroform, paraformaldehyde, guanidine thiocyanate, brine solution, Chlorox, methanol:chloroform:propionic acid, glutaraldehyde, formaldehyde, trichloroacetic acid or sodium cacodylate was degraded after one week at 37°C, whether extracted with or without Pronase E. However, some genomic DNA was obtained from the EDTA and 95% ethanol treatments (1 week) without Pronase E, and a yield equal to fresh spinach was obtained when Pronase E was used. After one month storage at 37°C, the DNA was degraded from both the EDTA and 95% ethanol treatments.

The yields of DNA from treatments using the alcohol series ranging from methanol to decanol revealed an interesting trend (Figure 1, A and B). Spinach DNA was well preserved after three months in methanol and ethanol and then showed an increasing degradation pattern with increasing sizes of alcohols (Figure 1A). This could be due to either the increasing sizes of the molecules, their decreasing polarity and/or the decreased ability of large alcohols

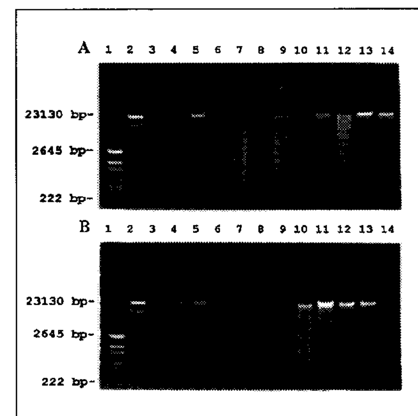

Figure 1. Effects of storage in various alcohols (100%) on yields of genomic DNA from spinach and broccoli. (Panel A) Spinach after 3 months: lane 1, pGEM® markers; lane 2, λ *Hin*dIII markers; lane 3, mouse DNA, 5 ng; lane 4, mouse DNA, 10 ng; lane 5, mouse DNA, 20 ng; lane 6, decanol; lane 7, octanol, lane 8, heptanol; lane 9, hexanol; lane 10, pentanol; lane 11, 2-butanol; lane 12, propanol; lane 13, ethanol; lane 14, methanol. (Panel B) Broccoli after 4.5 months: lane 1, pGEM markers; lane 2, λ *Hin*dIII markers; lane 3, mouse DNA, 5 ng; lane 4, mouse DNA, 10 ng; have 5, mouse DNA, 20 ng; lane 6, decanol; lane 7, octanol; lane 8, heptanol; lane 9, hexanol; lane 10, pentanol; lane 11, 2-butanol; lane 12, propanol; lane 13, ethanol; lane 14, methanol.

to denature and precipitate proteins (DNases in particular).

An even more striking pattern was found for broccoli buds stored for four and one-half months in the alcohols series (Figure 1B). Note the well-preserved DNA in the ethanol- and propanol-stored samples (Figure 2). However, very little DNA was preserved in broccoli using the methanol treatment in contrast to the case with spinach (Figure 1). For spinach, the maximum yield of genomic DNA was obtained from ethanol, then methanol storage, whereas for broccoli, the maximum yield was obtained from 2-butanol, then propanol and ethanol. This may relate to the physical structure of the plant materials. Spinach leaves are very thin with a maximum surface area, whereas broccoli buds are spherical with a minimum surface area for solvent penetration. In addition, the broccoli buds seem to be covered by a waxy cuticle that may favor the penetration of the more nonpolar 2-butanol over methanol. In both broccoli and spinach, the higher mol wt alcohols (octanol, decanol) were not effective preservatives.

For both broccoli and spinach, ethanol (100%)-preserved tissues yielded either moderate or considerable amounts of genomic DNA. This led us to examine the stability of DNA in plant materials stored in a series of different concentrations of ethanol. Solutions of 100%, 95%, 70%, 50% and 25% ethanol were used for the storage of spinach and juniper leaves. The highest yield of genomic DNAs was obtained from the leaves stored in 100% and 95% ethanol for both spinach and juniper (Figure 2). Storage

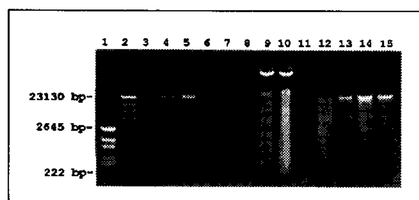

Figure 2. Yields of genomic DNA from spinach and juniper after 3 months storage in 25%–100% ethanol solutions. Lane 1, pGEM markers; lane 2, λ *Hin*dIII markers; lane 3, mouse DNA, 5 ng; lane 4, mouse DNA, 10 ng; lane 5, mouse DNA, 20 ng. Lanes 6–10 (juniper): lane 6, 25% ethanol; lane 7, 50% ethanol; lane 8, 70% ethanol; lane 9, 95% ethanol; lane 10, 100% ethanol. Lanes 11–15 (spinach): lane 11, 25% ethanol; lane 12, 50% ethanol; lane 13, 70% ethanol; lane 14, 70% ethanol; lane 15, 100% ethanol.

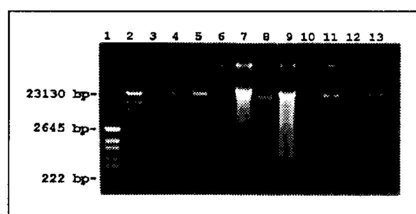

Figure 3. Effects of vacuum infiltration of ethanol (100%) into spinach and broccoli on yields of genomic DNA after storage in 100% ethanol for 11 months with and without the use of Pronase E (PE). Lane 1, pGEM markers; lane 2, λ *Hin*dIII markers; lane 3, mouse DNA, 5 ng; lane 4, mouse DNA, 10 ng; lane 5, mouse DNA, 20 ng. Lanes 6–9 (broccoli): lane 6, vacuum, without PE; lane 7, vacuum, with PE; lane 8, no vacuum, without PE; lane 9, no vacuum, with PE. Lanes 10–13 (spinach): lane 10, vacuum, without PE; lane 11, vacuum, with PE; lane 12, no vacuum, without PE; lane 13, no vacuum, with PE.

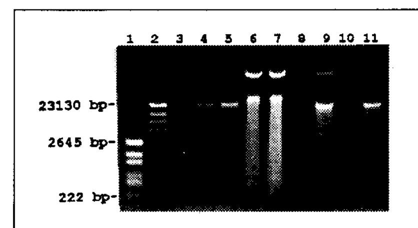

Figure 4. Effects of the use of Pronase E on the yields of genomic DNA from ethanol (100%)-preserved spinach (3 mos.), broccoli (4.5 mos.) and juniper (3 mos.). Lane 1, pGEM markers; lane 2, λ *Hin*dIII markers; lane 3, mouse DNA, 5 ng; lane 4, mouse DNA, 10 ng; lane 5, mouse DNA, 20 ng; lane 6, juniper without PE; lane 7, juniper with PE; lane 8, broccoli without PE; lane 9, broccoli with PE; lane 10, spinach without PE; lane 11, spinach with PE.

in 25% ethanol failed to preserve the DNA after one month in both spinach and juniper. The effects are even more pronounced in juniper leaves where the DNA was degraded in 25%, 50% and 70% treatments after three months (Figure 2). From these data, it appears that preservation in 95% ethanol is a more effective than 100% ethanol.

It would seem likely that rapid denaturation of DNases would be advantageous and facilitated by rapid infiltration of ethanol into cells. To test this hypothesis, vacuum infiltration of ethanol was performed on spinach and broccoli samples. Vacuum ethanol infiltration resulted in approximately twice the yield of genomic DNA, even after 11 months of storage (Figure 3). In addition, without the use of proteinase (Pronase E), essentially no DNA was obtained from spinach and very little from broccoli (Figure 3).

To examine the general effects of the use of Pronase E on DNA extraction, spinach, broccoli and juniper were stored in ethanol and then extracted with and without Pronase E. The use of Pronase E makes a large difference in DNA yields (Figure 4). This is suggestive that the ethanol complexes the protein to the DNA, so the DNA may be discarded with the protein and cellular debris during extraction. The failures of DNA alcohol preservation studies in the past seem to be not from the inability of alcohol to preserve the DNA effectively but from the failure to extract the bound DNA from the preserved sample. The addition of Pronase E seems to make it possible to extract high-quality DNA from preserved samples for up to (if not more than) 11 months of storage.

The literature is clear that variable yields of high molecular weight DNA are likely because of complexing DNA with phenolics/tannins as well as with polysaccharides either before or during extraction (2–7). This study suggests that another factor for low yields is the binding of DNA with proteins that prevent the extraction of DNA. This can be corrected by the addition of proteinase at the incubation/extraction phase. It is also possible that DNases are not fully inhibited with current protocols, and proteinase digests the DNases, which results in greater yields of genomic DNA.

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