

## SEVENTEEN YEARS STORAGE OF JUNIPER AND SPINACH LEAVES IN ALCOHOLS: EFFECTS ON DNA

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### ABSTRACT

Propanol appeared better than ethanol for the long-term storage of spinach leaves, followed by hexanol and pentanol. The lowest molecular weight and yields of DNA came from spinach stored in methanol and ethanol. In an experiment with spinach leaves stored in 100, 95, 70, 50 and 25% ethanol, the 50% ethanol stored leaves appeared to yield more and higher molecular weight DNA than any other treatment. In contrast, juniper leaves stored in 100 and 95% ethanol yielded more and higher molecular weight DNA than 70, 50 or 25% ethanol. The different manner that these two very different leaves respond to storage in ethanol solutions may reflect the herbaceous nature of spinach leaves versus the woody nature of juniper leaves, as well as differences in secondary compounds. *Phytologia* 93(3): 283-292 (December 1, 2010).

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Although silica gel is useful for the short-term preservation of leaves for subsequent DNA extraction for many plant species (see

Liston et al. (1990) apparently were the first to report on the utilization of silica gel in the field, although Doyle and Doyle (1987) earlier suggested that drying appears to be effective in preserving DNA. Silica gel is not very useful for some species such as ferns, which have very large amounts of tannins that turn the leaves yellow or brown during desiccation in silica gel (Thomson, 2002). The latter author also reported that a saturated NaCl-CTAB solution with 200 mM sodium ascorbate as an antioxidant was effective for interim preservation of DNA in Bracken fern.

One of the earliest studies on interim preservation was by Pyle and Adams (1989) in which they examined freezing, desiccation, air drying, and various liquids that are traditionally utilized to fix cell and chromosome structures (eg., Perfix preservative, paraformaldehyde, etc.). They found that none of the traditional liquids (including ethanol) preserved DNA for more than a few days. However, the same lab later found that these conclusions were invalid for ethanol (Flournoy et al., 1996). Apparently, several organic solvents result in the denaturation and precipitation of proteins, including DNases and histones. The histones are associated with DNA and when precipitated, bind to the DNA resulting in little or no DNA extracted. Flournoy et al. (1996) found that the use of proteinase digestion during grinding resulted in good DNA from short-term ethanol-preserved spinach and juniper. This information was utilized by Adams et al. (1999) in extraction of DNA from recalcitrant grasses (vetiver, wheat, maize, etc.). Field preservation of vetiver in silica gel proved effective for transport, but grinding in CTAB gave degraded DNA. However, Adams et al. (1999) found that grinding first in ethanol denatured the DNases, then CTAB extraction (with the addition of proteinase) resulted in good, genomic DNA. Fukatsu (1999) examined several organic solvents and found DNA to be well preserved in aphids (and their endosymbiotic microorganisms) in acetone, ethanol, 2-propanol, diethyl ether, and ethyl acetate for 6 months and for 2 years with acetone. King and Porter (2004) reported that ethanol was preferred for the preservation of ants for up to 6 months before extraction. Mandrioli (2008) published a useful review of DNA preservation methods in museum specimens. Some specimens of Hymenoptera yielded useful DNA after 35 years storage in 70% & 100% ethanol (at 4°C), and samples of Coleoptera had useful DNA 40 years after silica gel desiccation.

Dawson et al. (1998) reviewed several methods for the field preservation of marine invertebrate tissue and found that DMSO-NaCl (0.1 M Tris, pH 8.0, 0.02 EDTA, 0.02% [wt./vol.] CTAB in saturated NaCl to be useful; the solution was autoclaved and 20% DMSO, 0.002%  $\beta$ -mercaptoethanol and 0.25M disodium EDTA added) was the most useful and practical field method for DNA preseveration (tested for up to 28 months). The reader is referred to a recent, excellent review of tissue-preservation methods (Nagy, 2010).

In 1994, we preserved leaves of *Juniperus virginiana* and spinach in various concentrations of ethanol and various mono-hydroxy alcohols ranging from methanol to decanol. After 17 years of storage at lab temperature (~20°C), it seemed an opportune time to examine the DNA in these tissues.

## MATERIALS AND METHODS

DNA was extracted from juniper and spinach leaves (12-13 mg) by use of a Qiagen mini-plant kit as per manufacturer's instructions with the addition of 150 ug proteinase E (Sigma P6911) after the RNase incubation. Genomic DNA was visualized by agarose gel electrophoresis by mixing 3  $\mu$ l DNA extract, 3  $\mu$ l pGEM markers and 3  $\mu$ l  $\lambda$ HindIII, and loading 6  $\mu$ l on a 0.6% agarose gel, then running at 100 v for 20 min. The images were captured on a Kodak Gel Logic 100 Imaging System, and profile analysis was used to determine the modal DNA size and range of DNA sizes. The DNA from some samples was subjected to PCR amplification. ITS (nrDNA) and petN-psbM amplifications were performed in 30  $\mu$ l reactions using 6 ng of genomic DNA, 1.5 units Epi-Centre Fail-Safe Taq polymerase, 15  $\mu$ l 2x buffer E (petN-psbM) or K (nrDNA) (final concentration: 50 mM KCl, 50 mM Tris-HCl (pH 8.3), 200  $\mu$ M each dNTP, plus Epi-Centre enhancers with 1.5 - 3.5 mM MgCl<sub>2</sub> according to the buffer used) 1.8  $\mu$ M each primer.

## RESULTS

The gel of spinach stored 17 years in various alcohols (Fig. 1) shows a lack of preservation in methanol and ethanol, but a surprising amount of DNA present in the propanol-stored spinach.

A comparison of the effects of different alcohols on the preservation of DNA in spinach leaves (Table 1) shows that the greatest yields were in propanol, heptanol and hexanol.

It is surprising to find the moderate yields from storage in the larger alcohols (hexanol, heptanol). The lowest yields were from methanol and ethanol. The leaf disks stored in ethanol and pentanol had lost much of their structural features and disintegrated readily upon contact with a forceps.

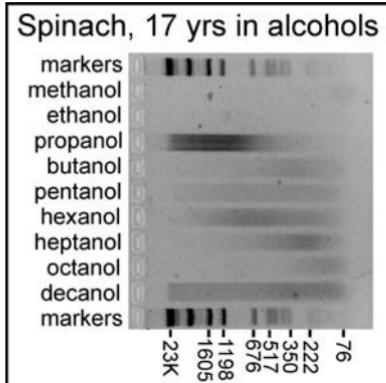


Fig. 1. Gel of spinach DNA stored 17 yrs. in various alcohols.

Table 1. Comparison of yield and DNA sizes from spinach after storage in various alcohols for 17b years.

alcohol	DNA yield (ng)	Mode(bp)	range (bp)
methanol	<0.3	1300	1650 - <76
ethanol	0.5	1000	1600 - <76
propanol	7.0	1200	~23K - 76
butanol	2.3	350	~2500 - <76
pentanol	2.3	500	~2500 - <76
hexanol	3.5	700	~2500 - <76
heptanol	3.7	250	~1500 - <76
octanol	2.3	150	~1500 - <76
decanol	3.5	300	~23K? - <76

DNA was scanned and profiles were obtained and compared with the markers (lambda\Hind III + pGEM) (Fig. 2). The patterns of degradation proved very different in the various alcohols (Fig. 2).

Propanol storage clearly yielded the most and highest molecular weight DNA (Fig. 2). A second trend is seen in the increase in molecular weight from butanol and pentanol to hexanol (Fig. 2). A

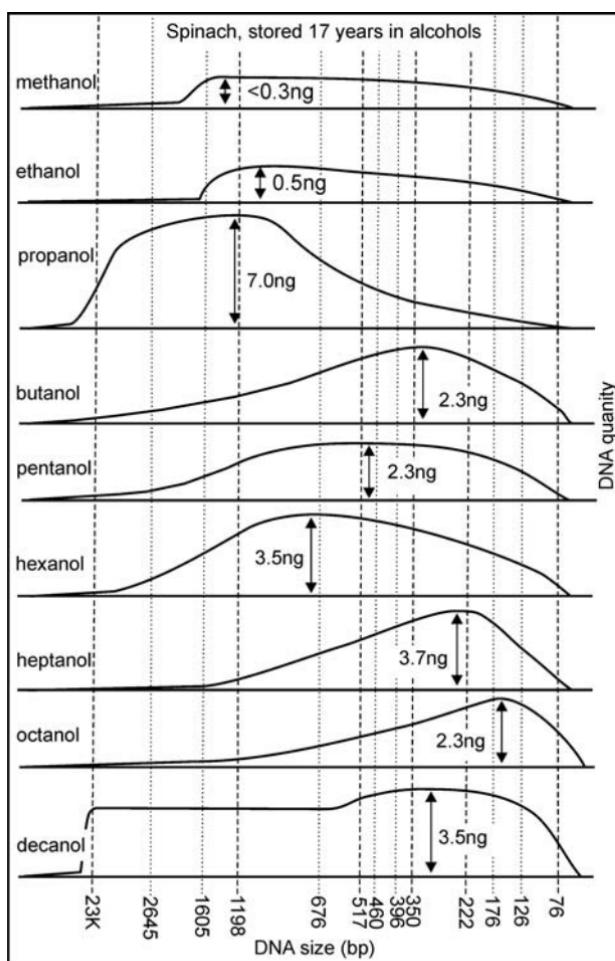


Figure 2. Profile analyses of DNA from spinach stored 17 yrs. in various alcohols.

third trend is the decrease in molecular weight from hexanol to heptanol. Finally, the last trend is the unusual curve for the decanol preserved spinach leaves. Although the mode is about 300 bp, there appears to be DNA as large as ~23K bp (Fig. 2). However, the curve is

quite flat from ~23KB to ~ 600 bp (Fig. 2), that is suggestive that some other kind of fluorescent materials may be responsible for this portion of the curve.

Figure 3 shows the almost complete loss of DNA at all concentrations except for the 50% ethanol treatment. This is surprising as this low concentration of ethanol does not seem to have been commonly utilized for preservation. Only the 100% ethanol treatment caused loss of structural integrity. It seems likely that 100% ethanol may dissolve the lipids in the membranes, as well as precipitation of the proteins, resulting in the loss of structure.

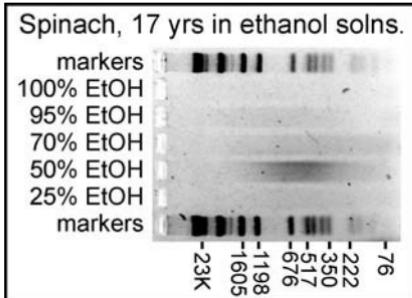


Figure 3. Gel of spinach DNA from leaves stored 17 yrs. in ethanol solns.

It should be noted that we (Flournoy et al. 1996) found large declines in high molecular weight DNA after 3 months storage in 25% and 50% ethanol. It was unexpected that the 50% ethanol storage solution was the best for spinach after 17 years. It may be, that for short-term storage (up to 3 mos.?), 100% EtOH is better but for long-term preservation of 1200-350 bp sized DNA (Flournoy et al. 1996), with 50% better for very long term storage (this study).

Flournoy et al. (1996) reported a decline in genomic DNA with the larger alcohols, with no visible DNA in a decanol solution after 3 months storage. In the current 17 year storage test, the presence of large molecules of fluorescent materials on the gel (Figs. 1, 2) may be due to plant secondary products that have cross-linked with degraded bits of DNA. Additional studies are in progress to investigate the nature of this material.

Profile analyses of spinach DNA stored in 100, 95, 70, 50 and 25% ethanol (Fig. 4) revealed that by far the most DNA was recovered from the 50% ethanol treatment. The 70% treatment is interesting in yielding a moderate amount of DNA (0.7 ng, Fig. 4) with a range from ~2500 bp to < 76 bp as well as material ranging from ~23K bp to 2500 bp. Prepping of the 'high molecular weight DNA' and PCR amplification is needed to confirm that such material yields useful DNA amplifications (in progress, RPA).

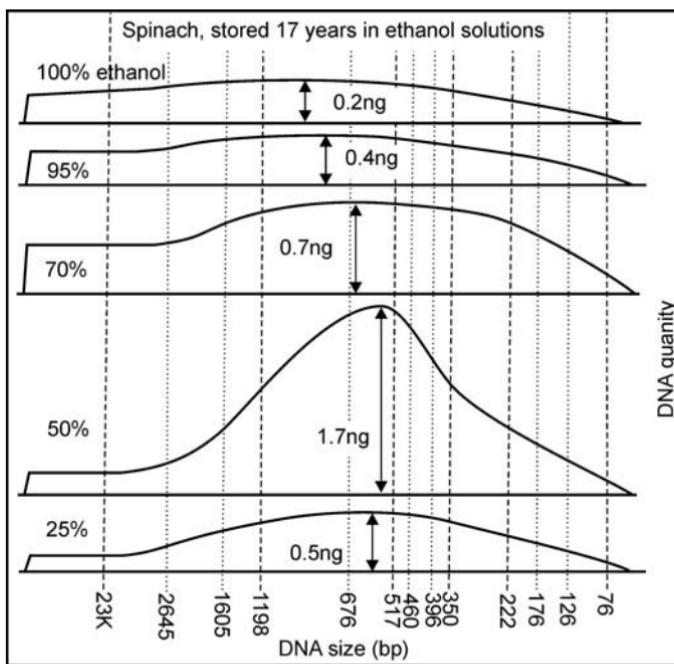


Figure 4. Profile analyses of DNA from spinach stored 17 yrs. in 100, 95, 70, 50 and 25% ethanol.

A gel showing the DNA of juniper leaves (*J. virginiana*) stored in different concentrations of ethanol (Fig. 5) reveals a very different pattern than seen with spinach (Fig. 4). Very little DNA was

obtained in 70 to 25% ethanol, with the most DNA obtained in 100% followed by 95% ethanol (Fig. 5).

No structural changes were observed in any treatment, this seems likely due to the woody nature of juniper leaves with hemicelluloses and lignans present. However, differences in chlorophyll color was quite obvious: 100% - bright green; 95% - bright green; 70% - pale green - yellow; 50% - very pale yellow-brown; 25% - very pale (nearly clear) brown.

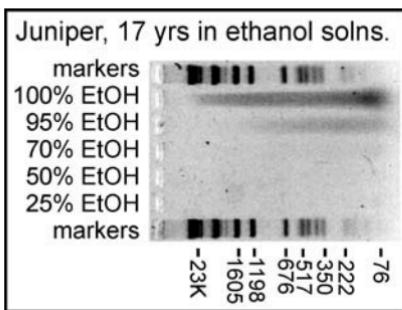


Figure 5. Gel of DNA from juniper stored 17 yrs. in 100, 95, 70, 50 and 25% ethanol.

Profile analyses (Fig. 6) of juniper leaves stored 17 yrs. in 100, 95, 70, 50 and 25% ethanol solutions had very different patterns than

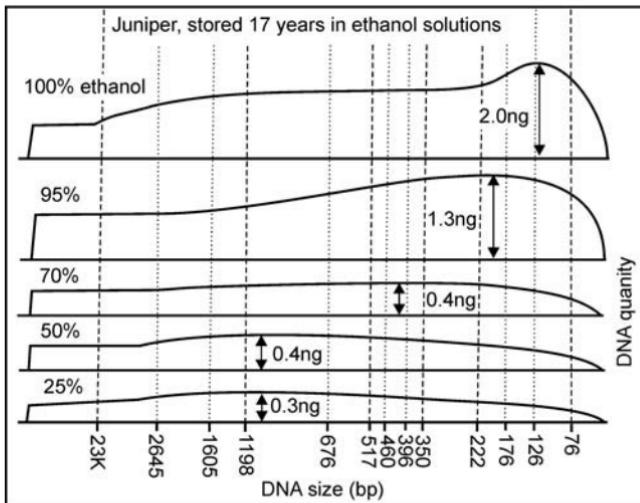


Figure 6. Profile analyses of DNA from juniper leaves stored 17 yrs. in 100, 95, 70, 50 and 25% ethanol.

seen with spinach leaves (Fig. 4). Both 100 and 95% ethanol preserved much more DNA than lower concentrations; however, there appears to be some larger DNA (2700 bp) in the 50 and 25% treatments (Fig. 6). Again, it is not known if this higher molecular weight material is useable DNA. PCR of ITS (nrDNA, ~1300 bp) was successful using DNA from the 95% treatment, but only barely successful for DNA from the 100% treatment. PCR of petN-psbM (cp DNA region, ~800 bp) was fair using DNA from 100%, and very poor for 95% treatment.

## CONCLUSIONS

Preliminary data suggest that propanol may be superior to ethanol for the long-term storage of spinach leaves, followed by hexanol and pentanol. Spinach DNA appeared to be least degraded when leaves were stored in 50% ethanol, but additional studies are needed to characterize the degraded materials. In contrast, the highest molecular weight DNA from juniper was obtained from 95 and 100% ethanol storage solutions. The different manner that these two species leaves respond to storage in ethanol solutions may reflect the herbaceous nature of spinach leaves versus the woody nature of juniper leaves, as well as differences in secondary compounds. Of course, none of the methods examined was nearly as effective in DNA preservation as desiccation followed by freezing. A interesting new paper (Akinnagbe et al., 2010) found that soaking of *Picea* leaves in 70, 80, 90 or 100% ethanol before desiccating in silica gel produced nearly twice the yield of DNA as desiccation with no pretreatment. No differences were found in DNA yields after soaking in ethanol for 24, 36, or 48 h. They hypothesized that ethanol-soaking before desiccation in silica gel may have deactivated DNases, disrupted cell walls and/ or extracted certain carbohydrates from the leaves. Such effects may be a factor in how alcohols act and need further investigation.

## ACKNOWLEDGEMENTS

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