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PRESERVATION OF DNA IN PLANT SPECIMENS FROM TROPICAL SPECIES BY DESICCATION

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Summary - The preservation of genomic DNA in spinach leaves by desiccation, using drierite and silica gel, was examined. Drierite and silica gel were found to be equally effective in preserving DNA in spinach leaves for up to 6 months at 37°C. Similar results were obtained using *Juniperus* leaves but *Magnolia* leaves had more degradation and oak (*Quercus*) leaves had considerable degradation after 6 months storage in silica gel at 37°C. Storage tests involving fresh and dried spinach leaves indicated that either materials can be used, but air dried spinach leaves had slightly less DNA degradation at each sample interval. The use of either silica gel or drierite appears to be an acceptable method for the preservation of DNA in tropical plant leaf specimens for subsequent extraction and archiving or analyses. A practical field protocol for DNA collection is presented.

INTRODUCTION

It is now estimated that the principal areas of diversity among plants, the lowland tropical forests, will have been cut over or severely damaged within the next 20 - 30 years (Raven, 1987, 1988). The diversity in these areas is immense: the Amazon River system, for example, contains eight times as many species as the Mississippi River system (Shulman, 1986). Raven (1987, 1988) estimates that approximately 60,000 plant species

(1 of 4) will go extinct with the loss of the tropical rain forests and as many as 1.2 million species (plants and animals) will go extinct in the next twenty to thirty years without factoring in the long-term losses due to global greenhouse effects. The extinctions of plant species mean the loss of thousands of links in plant phylogeny which will seriously hinder phylogenetic and evolutionary studies forever. The extinctions also mean a loss of valuable genes that could be put into our current and potential crop species by genetic engineering.

Relatively few scientists were interested in a 'genetic insurance policy' when the idea of banking genomic DNA from plants was first proposed (Adams, 1988). However, currently there are 30 institutions (representing 21 nations and every continent) in DNA Bank-Net (Figure 1).

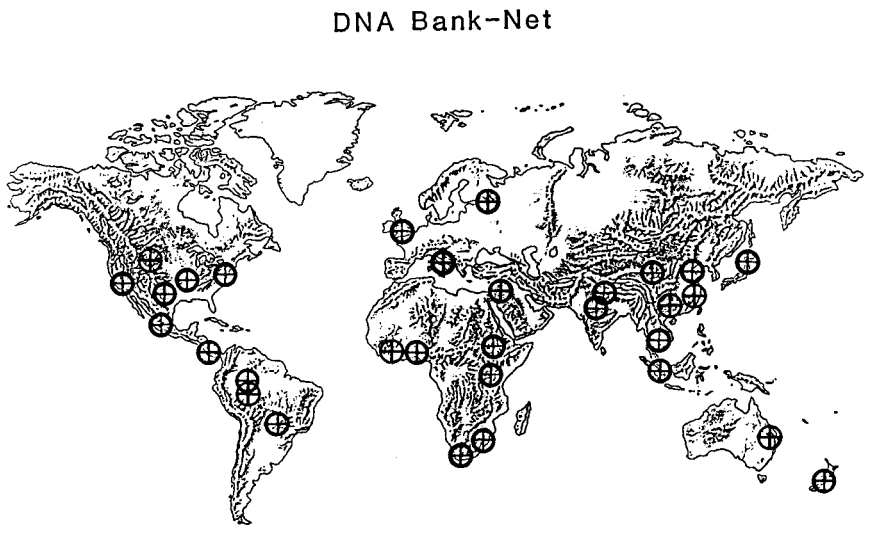

Figure 1. Map of cooperating and projected members of DNA Bank-Net. Additional nodes in the DNA Bank-Net will be added as the association develops.

Collections of plant specimens have been utilized for the formulation of our understanding of morphological variation among taxa. Indeed, without the great herbaria of the world, our knowledge of plant evolution would be fragmented at the least. As we have moved into the era of utilizing chemical data for systematic and evolutionary studies, methods of preserving plant materials for future (chemical) work have been largely ignored. We are usually content to file a voucher specimen to document our chemical studies. With the present level of support for plant collections it is unlikely that much of the world's plant species can be preserved by freezing so that scientists might have access to the study of secondary compounds, enzymes, or DNA/RNA in the coming centuries.

The problems associated with bringing back fresh or frozen materials can generally be overcome by specialists (ex. worldwide collections of fresh foliage of *Juniperus* for essential oil analyses and DNA by RPA). However, botanists doing floristic research will collect the bulk of the specimens from tropical rare and endangered species. They often collect specimens from scores of different species in a single day. The bulk of the materials that they have to process and ship requires that any protocol for the collection of samples for specialized needs (ex. DNA storage/analyses), must be quick, simple and trouble-free. The generalist collector, working in tropical areas cannot be expected to preserve hundreds or thousands of collections for months under tropical conditions and then arrange transport through customs, all the while keeping the individual specimens frozen.

Fortunately, at least as far as DNA preservation is concerned, the ancient Egyptians provided a clue to the solution. Paabo (1985) reported that cloned DNA from dehydrated mummy tissues (2,400 y old) "seemed to contain little or no modifications introduced postmortem". He further postulated that the use of crystalline salts for desiccation prevented the normal hydrolytic processes (DNase activities). DNAs from the more readily dehydrated tissues (skin, outer ear) were much better preserved than tissues from internal organs that were more difficult to dehydrate (Paabo, 1985). DNA has also been obtained from the extinct quagga (dried muscle attached to

salt-preserved skin, Higuchi *et al.*, 1984), a 5000 year old Egyptian mummy (Paabo, 1989) and a 7000 year old brain (Paabo *et al.*, 1988) preserved in a peat bog.

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 from 20 to 95 years old. DNA from *Juniperus osteosperma* seeds from pack rat middens (3,500; 27,000; and greater than 45,000 y bp) yielded DNA with a maximum lengths of 10, 10, and 10 kbp and average lengths of 7, 5, and 3 kbp, respectively.

Doyle and Dickson (1987) attempted 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. Although none of the chemical treatments preserved DNA for more than a few weeks, Doyle and Dickson (1987) did obtain adequate sized DNA from air dried leaves.

Dally and Second (1989) reported that dry leaf powder could be stored for many months over P_2O_5 (a very powerful desiccant) at -20°C. Unfortunately, no data was presented about storage at higher temperatures as encountered when shipping materials by surface freight both within and between tropical countries. Nor did Dally and Second (1989) comment on the use of a safer desiccant than the highly corrosive P_2O_5.

Pyle and Adams (1989) examined the use of acetic acid/ethanol (1:3), ethanol (95%), glycerol, Na azide (3%), Perfix preservative, pentachlorophenol/chloroform (1.5/98.5), paraformaldehyde (10%), guanidine thiocyanate (1%), NaCl (25%), Chlorox (5%), methanol/chloroform/propionic acid (1:1:1), glutaraldehyde (8%), formaldehyde (7.4%), trichloroacetic acid, (10%), glutaraldehyde (2%)/0.5M Na cacodylate and 10mM EDTA to preserve spinach leaves. None of the aforementioned solutions preserved the DNA in spinach for 7 days (Pyle and Adams, 1989). However, the preservation of DNA by desiccating leaves in silica gel or drierite was reported by Pyle and Adams (1989). They obtained high molecular weight

DNA from spinach following desiccation and storage over drierite in a desiccator after 2 months but the DNA was degraded when tested at 5 months (see also Adams, 1990).

Liston, *et al.* (1990) reported good DNA yields from numerous specimens from Xinjiang, China that had been field preserved in drierite. Chase and Hills (1991) used silica gel to preserve leaves of *Ericaceae* and *Malpighiaceae* species with success. Both of these studies analyzed the DNA after only a few weeks in the desiccant. No long term studies have been reported to date.

Those results prompted us to perform additional storage tests in our lab using both fresh and air dried leaves of spinach, juniper, magnolia, and oak by placing the leaves directly in contact with silica gel and drierite in jars. In addition, both drierite and silica gel were further investigated in regards to drying efficiency and capacity.

PREPARATION OF PLANT MATERIAL

Leaves from *Juniperus virginiana* L. and *Quercus virginiana* Mill. were collected from native trees on the Baylor University campus. *Magnolia grandiflora* L. leaves were collected from cultivated trees on the Baylor campus. Leaves from fresh spinach (*Spinacia oleracea* L.) were purchased locally. Approximately 0.5 g (fw) of leaf was used for each storage treatment. Leaf drying was done in a conventional plant press dryer at 42°C for 24 - 48 h. A few indicating crystals were mixed the drierite (anhydrous $CaSO_4$, W. A. Hammond Drierite Co.) or silica gel prior to drying. This allows visual inspection of the jars in storage as the blue crystals turn pink when hydrated. A jar with a cracked or loose cap, for example, can be easily detected. Desiccation was performed by half filling a 30 ml screw capped glass jar with drierite or silica gel, adding the leaf, and then completely filling the jar with the appropriate drying agent. The jars were stored in an incubator at 37°C until analyzed (in triplicate).

For the experiments on the determination of the drying rates for drierite and silica gel, fresh spinach leaves were cut into 2 cm x 2 cm squares (0.5 g FW) and placed in jars with 20 g of drierite or silica gel at 22°C and sealed. After

24, 48, 72, 96 or 120 h, the leaves were taken from 3 jars of both drierite and silica gel, weighed, then dried in a microwave oven for 8 min (700 watts) and weighed immediately. Preliminary experiments showed that 4 min at 700 watts in the microwave was equivalent to oven drying at 100°C for 48 h. To be extra careful, the leaves were dried an additional 4 min (8 min total) in the microwave on high (700 watts). Percent dry matter was calculated as: 100 x dry weight / weight before drying.

DNA EXTRACTION AND ANALYSES

The hot CTAB procedure (Doyle and Dickson, 1987; Doyle and Doyle, 1987) was used for DNA extraction with minor changes as noted in Pyle and Adams (1989). Studies (Birren *et al.*, 1989; Bostock, 1988; Carle, Frank and Olson, 1986; Gekeler *et al.*, 1989; Devos and Vereruysse-Dewitte, 1989) on the separation of high MW DNA (up to several hundred kbp) by field inversion gel electrophoresis (FIGE) prompted us to use FIGE for the analyses of genomic DNA in this study. The extracted DNAs were run on a 1% agarose gel, using a BioRad mini-sub electrophoresis unit, and a BioRad Pulsewave 760 to invert the current (70 v), starting at 3 sec forward and 1 sec reverse, ramped to 12 sec forward and 4 sec reverse at the end of 4 h. T5 DNA (Sigma D8010, 103 kbp), Lambda DNA (Sigma D9768, 48.5 kbp) and HindIII restricted lambda DNA (23.13, 9.614, 6.557, 4.361, 2.322 and 2.027 fragments used) were co-run to provide size standards. Two µl of a 20 ng/µl fluorescein isothiocynate dextran (Sigma FD2000S, 2 million MW) solution was added to each well just prior to photographing in order to clearly delineate the front of the wells on the densitometer scans. The Gels were photographed under short wave UV light using a Polaroid direct screen camera (DS34). DNA was quantified by use of a video densitometer (JAVA video analysis system, Jandel Scientific Inc.; CCD video camera, model WV-BL200, Panasonic Corp.; PC Vision frame grabber, Image Technology, Inc.). Average and mode molecular weights and the percent (%) of the DNA greater than 6 kbp were calculated using program GRAFGEL (program available for IBM PC from RPA). Restrictibility of stored DNA was checked using EcoRI, Hind III, Kpn I,

and Sal I (Sigma R2132, R1882, R9506 and R0754, respectively).

RESULTS AND DISCUSSION

The storage of fresh spinach leaves (figure 2) in silica gel for up to six months appears to be satisfactory. Notice

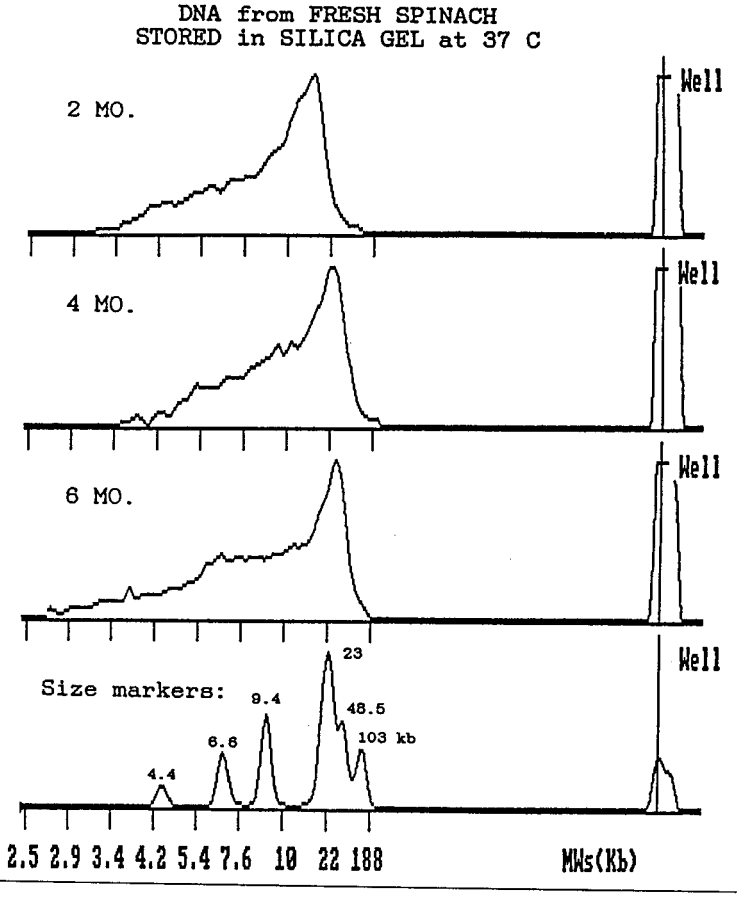

Figure 2. Video densitometer scans of FIGE of genomic DNA from fresh spinach leaves stored in silica gel at 37°C for 2, 4 and 6 months. Notice the gradual increase in lower molecular weight DNA (the peak tailing to the left). Size markers are : lambda DNA cut with HindIII (2.3, 2.0 and 0.5 kbp bands not shown); Lambda DNA (48.5 kbp) and T5 DNA (103 kbp).

that the genomic DNA ranges from about 190 kbp down to about 2 kbp (after 6 mos., Figure 2). The mode and average MWs (in kbp) were: 2 mo.- 13.4, 10.7; 4 mo.- 24.5, 18.6; and 6 mo. -29.7, 16.5. The only explanation we have for the increasing mode and average MWs with time in storage is that several

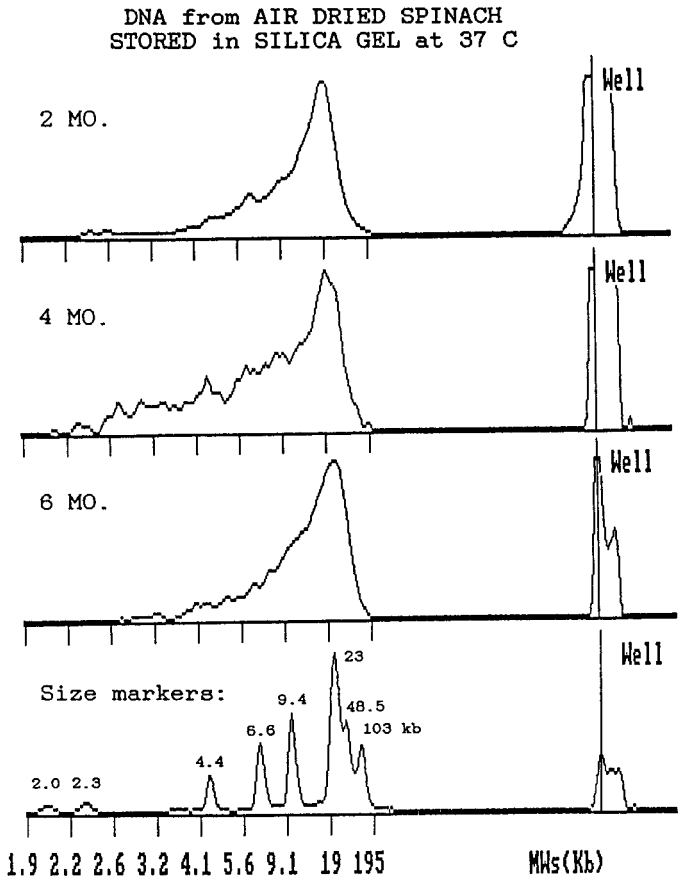

Figure 3. Video densitometer scans of FIGE of DNA from air dried spinach leaves stored in silica gel at 37°C for 2, 4 and 6 months. Size markers are: lambda DNA cut with HindIII (0.5 kbp band not shown); Lambda DNA (48.5 kbp) and T5 DNA (103 kbp).

changes in the protocols were made (such as cutting off the Eppendorf pipet tips before transferring DNA) during the span of this research. It seems likely that the increases in the mode and average molecular weights were due to improvements in techniques during the experiment.

The DNA in the air dried spinach leaves appears similar to DNA from fresh leaves (Figure 3). The mode and average MWs increased in the 6 mo. sample (mode, average MW in kbp: 2 mo. - 15.2, 15.1; 4 mo. - 15.2, 12.2; 6 mo. - 19.2, 16.1) just as with the fresh spinach. One might note that the densitometer tracing for the 4 mo. sample (Figure 3, second scan) displays a rather jagged trace from 2 to 10 kbp. This due to the sample being under-loaded on the gel (i.e., high background noise to signal ratio). The trace for the 6 mo. sample has much less noise due to a higher sample loading level (DNA was more concentrated in the sample). Comparisons of three different storage tests are shown in Figure 4. None of the stored leaves had as much high molecular weight DNA as fresh spinach, but the DNA from air dried leaves, stored in silica gel at 22^0 for 8 months, was quite comparable. The lower storage temperature (22^o vs. 37^oC) appears to make a difference in the average molecular weight.

Analyses of the effects of two storage temperatures (22^oC and -20^oC) on the DNA in air dried spinach leaves stored in silica gel or paper envelopes gave the following results (after 8 mo. storage):

Treatment	Avg. MW(kbp)	Mode MW(kbp)
silica gel, -20^oC	31.5	27.7
silica gel, 22^oC	28.6 kbp	33.2
envelope, -20^oC	34.0	29.2
envelope, 22^oC (1 mo.)	< 1 kbp	< 1 kbp

It should be noted that during the first month of storage, we experienced almost continual rainfall for two weeks and the ambient relative humidity in the laboratory was very high (90 -100%). Apparently the paper envelopes absorbed ambient moisture and rehydrated the leaves, leading to DNA breakdown.

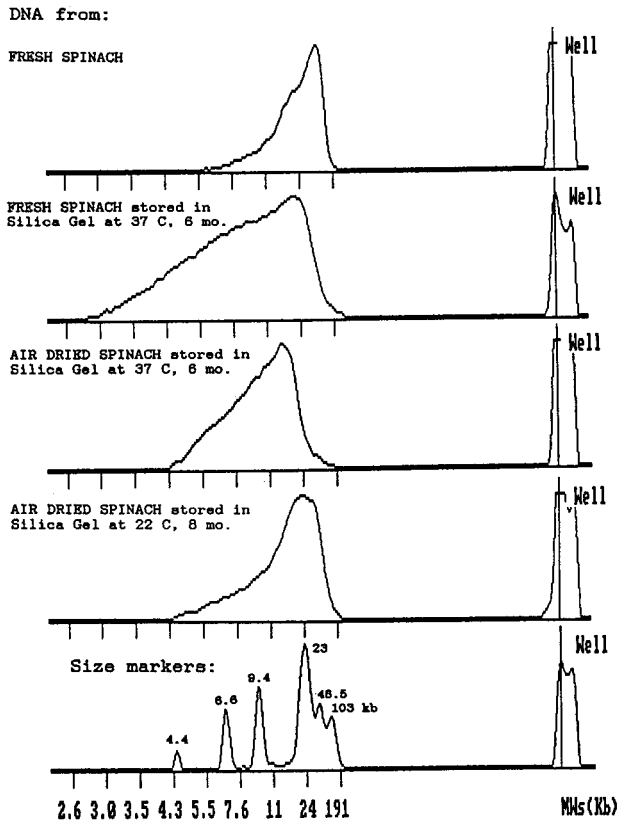

Figure 4. Video densitometer scans of FIGE of genomic DNA from: fresh spinach leaves; fresh spinach leaves stored in silica gel at 37°C for 6 months; air dried spinach leaves stored in silica gel at 37°C for 6 months; and air dried spinach leaves stored in silica gel at 22°C for 8 months. Size markers are: lambda DNA cut with HindIII (2.3, 2.0 and 0.5 kbp bands not shown); Lambda DNA (48.5 kbp) and T5 DNA (103 kbp).

There were no significance differences between any of the average and mode MWs for the silica gel, -20°/22°C or envelopes, -20°C treatments. However, due to the small number of replicates (3) per treatment, one must view these results as

preliminary.

Results similar to spinach were obtained using fresh leaves of *Juniperus virginiana*. However, the DNA from magnolia (*Magnolia grandiflora*) leaves showed considerably more degradation after 6 months at 37°C than spinach and the DNA from liveoak (*Quercus virginiana*) was even more degraded. The problems with the storage of magnolia and liveoak leaves are not understood at present and additional research is being conducted. It is possible that interfering cellular components (such as polysaccharides, see Do and Adams, 1991) form complexes or lipids produce free radicals that degrade the DNA during storage and/or extraction. The thick waxy cuticles on liveoak and magnolia may have slowed the escape of moisture, leading to conditions favoring DNA degradation. In any case, avoiding high storage temperatures is beneficial and one should place the materials into a freezer as soon as they are received.

Generally, the DNA from desiccated fresh spinach shows more small (2 - 6 kbp) DNA fragments than the DNA from desiccated air dried spinach leaves. We can only speculate that some DNase activity occurs in the fresh leaves before the silica gel effectively dehydrates the leaves and denatures the DNases. Labuza (1970) reviews the properties of water in food preservation and states that 'water of natural foods does not leak out unless some damage occurs to the tissues'. It is possible that the desiccation of fresh spinach in silica gel is too severe and membranes are lysed, allowing DNases access to compartmentalized DNA. On the other hand, air drying (see methods) may lead to more volatilization of water with less membrane damage. Even bound water (0.05 - 0.1 g H_2O per g dry wt) cannot be regarded as unavailable. Duckworth and Smith (1963) demonstrated that glucose, calcium chloride and sulfate migrated along food surfaces even at monolayer water values. These water activities (a_w = 0.2 - 0.3) were so low that, theoretically, water could not act as a solvent (Labuza, 1970). So it does appear that some degradative action may be occurring, even in desiccated materials. The problem of preserving specimens can be related to water activity (a_w). It is well established that the lower limits for growth of bacte-

ria is a_w = 0.9 to 0.75 and for fungi 0.60, with no sustained growth of any known organism below a_w =0.60 (Bone, 1969). Labuza (1970) shows enzymatic activities approaching zero rates at a_w = 0.3, with non-enzymatic browning occurring down to a_w = 0.2 and lipid oxidation reaction rates minimized at a_w = 0.3, then **increasing at lower water activities!**. Unfortunately, research is needed to relate these principles to the preservation of DNA *in situ*.

The choice of desiccant was investigated and narrowed to drierite and silica gel. Although there are a number of compounds that are more powerful desiccants (such as P_2O_5), these compounds are so reactive with water to render them dangerous for routine field usage and particularly troublesome when trying to negotiate entry or exit with customs officials. Both

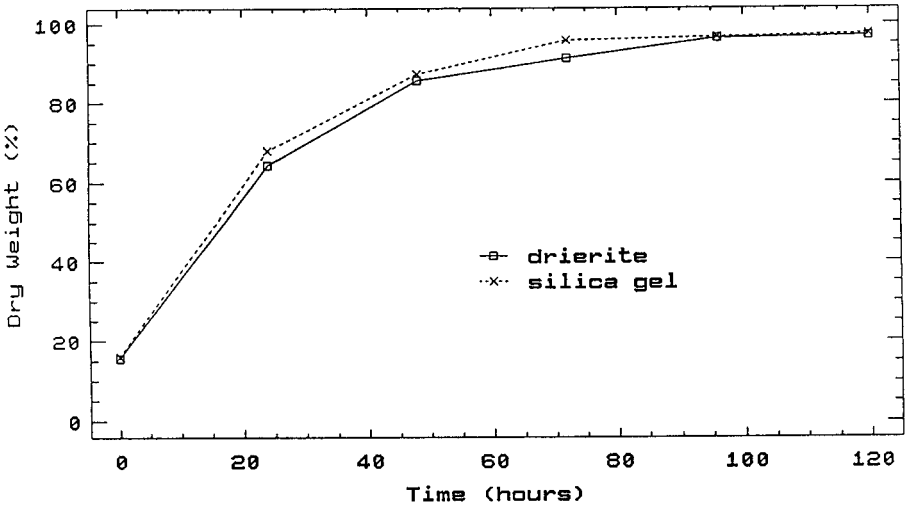

Figure 5. Changes in percent dry matter for fresh spinach leaves stored in drierite or silica gel. No significant differences were found between drierite and silica gel for any particular time period sample. See Table 1 for significance tests between time periods for both drierite and silica gel treatments.

drierite and silica gel are light, inert and inexpensive. Tests of leaf drying (Figure 5) revealed that percentage of dry matter asymptotes after 96 h at 96 to 97% dry matter (for spinach) for both desiccants. Notice that the curves for both drierite and silica gel are very similar. No significant differences in the percent dry matter were found between storing samples in drierite or silica gel at any given time period. No significant differences in the percent dry matter were found after 72 h storage in drierite or silica gel (Table 1).

The dry matter values for spinach leaves compare with a 98.1% dry matter obtained by drying the moss, *Tortula ruralis* with silica gel (Schonbeck and Bewley, 1981) and 96.4% dry matter when *Selaginella lepidophylla* fronds were dried in

Table 1. Changes in dry weight for fresh spinach leaves when stored in drierite or silica gel. All storage tests were performed at 22°C. Any two means sharing a common superscript are not significantly different by the SNK (Student-Newman-Keuls) multiple range test, p = 0.05, based on 3 replicates.

	% dry weight after storage in	
Time stored	Drierite	Silica gel
Fresh (initial)	15.65[a]	16.05[a]
24 hours	64.28[b]	67.90[b]
48 hours	85.57[c]	87.27[c]
72 hours	91.23[c,d]	95.73[d]
96 hours	96.17[d]	96.52[d]
120 hours	96.77[d]	97.27[d]

drierite (Eickmeier, 1988). The relative humidity of drierite is estimated at 1% and the water potential at -600 MPa (Eickmeier, 1988), compared to ca. 1% and -6000 MPa, respectively, for silica gel (Schonbeck and Bewley, 1981). Wiebe (1981) found *Tilia americana* leaves dried in a conventional plant press reached a water activity (a_w) of 0.5 (-100 MPa), whereas oven dried leaves (temperature and time not reported) had an a_w of 0.25 - 0.35 (-150 to -200 MPa) and ground leaves, stored over silica gel had an a_w of 0.06 (-400 to -500 MPa). Based

in Wiebe's graph of water potential and a_w (Wiebe, Figure 4, 1981), it appears that drierite is comparable to silica gel in desiccating power. Thus, it would seem that the levels of water activity attained by the use of drierite or silica gel (a_w less than 0.06) should stop both enzymatic and factors such as non-enzymatic browning but not stop lipid oxidation (Labuza, Figure 20, 1970). However, one should bear in mind that leaf materials are hydroscopic, so it is unlikely that the leaves reached the a_w of drierite or silica gel.

PROTOCOL FOR FIELD PRESERVATION OF FOLIAGE

Drierite has a water capacity of 10 to 14 percent, but above 6.6%, the capacity varies inversely with temperature (W. A. Hammond Drierite Co.). One would not want to risk possible rehydration of leaves, so storage ratios should be based on the 6.6% capacity. In lab tests, silica gel absorbed 8.85% of its weight of water after exposure to 100% humidity for 16 h at 22°C. We have found that plant materials contain as much as 92% moisture, so a useful approximation would be to assume the plant is mostly water and use 16 to 20 times the fresh leaf weight for the drierite or silica gel component.

Now that inexpensive ($100 USCY) battery powered, portable balances are available, one could take a supply of jars that hold (for example) 100 g of silica gel and then weigh out 5 g of fresh leaf material and add it to the jar along with silica gel (or drierite). We have found that air dried leaves (suitable for herbarium vouchers) generally contain from 10 to 15% water. Using a robust value of 20% water for air dried leaves, one can weigh out 5 g of air dried leaves (5 g x 20% = 1 g water) per 20 g of silica gel. This procedure may seem time consuming, but in practice, we merely do a quick check on the leaf area needed to give approximately 1 g (fresh leaves) or 5 g (dried leaves) and then just use that amount of leaf area. For example, for spinach, a 2 cm x 2 cm fresh leaf area weighs about 0.5 g. So, one can just cut the leaves into roughly 2 cm x 2 cm squares and add one square to 10 g of silica gel (0.5 g leaf / 10 g silica gel = 1/20 ratio). For succulent leaves, a slightly different protocol may be used. Liston *et al.* (1991) removed succulent leaf material after 24

hours in drierite and placed it in fresh drierite. A note of caution is necessary concerning field drying of specimens for subsequent silica gel/ drierite storage. We have experienced difficulty obtaining DNA from leaves dried at temperatures higher than about 55°C. In very rainy conditions where high drying temperatures (from butane stoves, for example) are used to dry specimens, it would seem advisable to merely blot leaves free of surface moisture and then place the fresh leaf material directly into silica gel or drierite. Liston *et al.*, (1991) took 2-5 g of plant tissue and wrapped it in tissue paper to prevent it from fragmenting, then placed it in a 125 ml Nalgene bottle, 1/3 prefilled with drierite (with blue indicator crystals), and then filled the bottle (2/3) with additional drierite.

Plastic bottles are probably to be preferred to glass, to avoid breakage in transit. Using clear jars allows one to check the indicating crystals without opening the jar. The lids should be sealed with vinyl tape to insure against moisture leakage. The use of parafilm to seal containers is not recommended, as we have found it to come loose at 37°C (and of course, at tropical temperatures!).

Silica gel and drierite do differ in one characteristic that may be a consideration. We have found that silica gel can be dried (recharged) at 100°C for 24 h but drierite must be dried at much a higher temperature (we had to use 200°C). In addition, we could easily dry silica gel in a microwave oven, but were unable to dry (recharge) drierite in a microwave oven. If the desiccant gets wet before use, silica gel appears to be much easier to dry. Silica gel is used in large quantities for flower drying and, thus, may be cheaper, depending on your source. Neither drierite nor silica gel should be discarded but recharged for reuse on subsequent trips. If the materials are to be checked through customs, it is useful to have a small container of silica gel/ drierite that you can open and show the customs agents. A demonstration that the blue indicator crystals will turn pink when you breath on or moisten them is helpful in convincing the customs officials to **not** open your sealed specimen jars.

In conclusion, we have found that both fresh and air dried

leaves can be preserved in drierite or silica gel for up to 6 months at tropical temperatures (37°C) so plant materials can be shipped by surface freight for the eventual extraction of DNA. This should greatly facilitate the collection of materials since one will not have to use expensive and complex freezing equipment in the field.

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