Storage effects on genomic DNA in rolled and mature coca leaves

Emanuel L. Johnson, Soo-Hyung Kim, and Stephen D. Emche
USDA ARS Alternate Crops and Systems Laboratory, Beltsville, MD, USA


Rolled and mature leaf tissue was harvested from Erythroxylum coca var. coca Lam. (coca) to determine a method for storage that would maintain DNA with high quality and content up to 50 days. Harvesting coca leaf tissue under Andean field conditions often requires storage from 3 to 10 days before extraction where tissue integrity is lost. All samples of rolled and mature coca leaf tissue were harvested and separately stored fresh in RNAlater™ for 50 days at 4°C, -20°C, and 23°C, while similar samples were air-dried for 72 h at 23°C or oven-dried for 72 h at 40°C after storage, before extraction. Triplicate samples of each tissue type were extracted for DNA at 10-day intervals and showed that DNA integrity and content were preserved in leaf tissue stored at 4°C and -20°C for 50 days. Rolled and mature leaf tissue stored at 4°C, -20°C, and 23°C showed insignificant degradation of DNA after 10 days, and by day 50, only leaf tissue stored at 4°C and -20°C had not significantly degraded. All air- and oven-dried leaf tissue extracts showed degradation upon drying (day 0) and continuous degradation up to day 50, despite storage conditions. Amplified fragment length polymorphism analysis of DNA from rolled and mature leaf tissue of coca stored at 4°C and -20°C for 0, 10, and 50 days showed that DNA integrity and content were preserved. We recommend that freshly harvested rolled or mature coca leaf tissue be stored at 4°C, -20°C, and 23°C for 10 days after harvest, and if a longer storage is required, then store at 4°C or -20°C.

INTRODUCTION

Research in our laboratory requires the extraction and isolation of genomic, mitochondrial, and chloroplast DNA from leaf tissue of several drug crop plants to determine inter- and intravarietal differences among and within taxa. Many of the taxa under investigation are indigenous to tropical environments and pose the problem of maintaining high-quality RNA and DNA during specimen collection and transportation. This is particularly true for taxa of Erythroxylum where the obstacles demarcated by Plowman and Rivier (1) still exist today. Several methodologies are available that address the preservation of plant material for molecular systematic studies for plants indigenous to tropical regions (2–6) as well as descriptions of their performance (5,7,8). The most efficient method for preserving plant material is with the use of liquid nitrogen, but this imposes inherent problems in tropical environments (2).

The genus Erythroxylum, which comprises 240 species, has four cultivated taxa (Erythroxylum coca var. coca Lam; Erythroxylum coca var. ipadu Plowman; Erythroxylum novogranatense var. novogranatense [Morris] Hieron; Erythroxylum novogranatense var. truillense [Rushy] Plowman) and is purported to contain many of the secondary allelochemicals found in Solanaceae (9). In addition, there are high contents of polyphenols in the leaf tissue (unpublished data); thus, during extraction, DNA is subjected to binding and degradation by these terpenoids and tannins (10). The difficulty of isolating high-quality RNA and DNA from highly phenolic plant tissue was reported by Katterman and Shattuck (11) and from recalciitrant plant tissue by Baker et al. (12). In Gossypium hirsutum of Malvaceae and the related family Bombacaceae, both phenolic terpenoids and tannins have been shown to bind to RNA and DNA after cell lysis, causing their degradation (10). Once combined with RNA or DNA, the phenolics cannot be removed by conventional extraction procedures, rendering them useless for in vitro translation or cDNA cloning studies (10). It has been shown that RNAlater™ successfully preserves RNA in plant tissue (13), and in this study we investigated whether it would also preserve DNA. To circumvent interference caused by polyphenolic terpenoids and tannins, highly alkaline pH buffering and polyvinylpolypyrrolidone (PVPP) were used in this study, which through hydrogen bonding sequestered the phenolics and tannins, therefore efficiently removing them from the leaf tissue homogenate as previously described (10,14,15). In a previous study, we successfully differentiated the Erythroxylum taxa using amplified fragment-length polymorphism (AFLP) DNA analysis (16). The purpose of this study was to find a method of storage for E. c. var. coca leaf tissue so that the content and integrity of the DNA of interest would be preserved so that it would be useful for future interspecific, intraspecific, and related DNA studies.

MATERIALS AND METHODS

Harvest and Storage of Leaf Tissue

Rolled (<10 days old from bud break; immature) and mature (>30 days old) leaf tissue of E. c. var. coca Lam. grown under greenhouse conditions at Beltsville Agricultural Research Center (Beltsville, MD, USA) (17) were separately harvested and stored at 0°C. A portion of rolled and mature leaves (3 samples/leaf type/storage condition) were extracted for genomic DNA (total DNA) using a DNeasy™ Plant Mini Kit (Qiagen, Valencia, CA, USA; procedures described below) and reported as day 0. All other rolled and mature leaves (4 and 2 leaves/tube, respectively) were separately submerged in 3.5 mL RNAlater (Ambion, Austin, TX, USA) in polyethylene tubes and stored up to day 50 after harvest under the following conditions: 23°C (room temperature); 4°C; -20°C; 23°C, oven-dried for 72 h at 40°C before extraction; and 23°C, air-dried for 72 h at 23°C before extraction. Rolled and mature leaves (3 sample replicates/storage condition) were removed on days 0, 10, 20, 30, 40, and 50 and extracted to determine DNA content and quality.
Table 1. Sequences of Primers Used for Selective Amplification of 4°, -20°, and 23°C (0-, 10-, and 50-Day) Samples

<table>
<thead>
<tr>
<th>Primer Pair</th>
<th>Forward Primer (EcoRI)</th>
<th>Reverse Primer (MseI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5′-D4-GACTGCGTACCAATTCACT-3′</td>
<td>5′-GATGAGTCCTGAGTAACAT-3′</td>
</tr>
<tr>
<td>2</td>
<td>5′-D4-GACTGCGTACCAATTACA-3′</td>
<td>5′-GATGAGTCCTGAGTAACAA-3′</td>
</tr>
<tr>
<td>3</td>
<td>5′-D2-GACTGCGTACCAATTCAAC-3′</td>
<td>5′-GATGAGTCCTGAGTAACAC-3′</td>
</tr>
<tr>
<td>4</td>
<td>5′-D3-GACTGCGTACCAATTCAG-3′</td>
<td>5′-GATGAGTCCTGAGTAACTA-3′</td>
</tr>
<tr>
<td>5</td>
<td>5′-D4-GACTGCGTACCAATTACC-3′</td>
<td>5′-GATGAGTCCTGAGTAACCTA-3′</td>
</tr>
</tbody>
</table>

Unique sequences and dyes are shown in bold type.

Samples were prepared for analysis by diluting the final amplified product 1:30 (v/v) in commercial Sample Loading Solution, which included 1% (v/v) 400-bp DNA size standards (both from Beckman Coulter). Fragment separation and detection were performed by a genetic analysis system (CEQ 8000™; Beckman Coulter); this used capillary electrophoresis on each sample, beginning with a 30-s electrophoretic injection at 2 kV and 35 min separation at 50°C and 6 kV.

Statistical Analyses

Analysis of variance was used to determine the effects of leaf tissue, storage temperature, storage treatment, and different storage days using SAS PROC GLM (SAS Institute, Cary, NC, USA). Orthogonal contrasts were constructed to test various hypotheses assessing the effects of various treatment combinations at selected days of storage (10 and 50 days). The hypotheses tested using the orthogonal contrasts were (i) leaf tissue, whether rolled or mature, does not have impact on genomic DNA content (rolled vs. mature); (ii) post-storage treatment does not alter the genomic DNA content (air dry vs. oven dry); (iv) different storage days using SAS PROC GLM; (v) the storage temperatures of -20°C and 4°C lead to no difference in DNA content (air dry vs. oven dry); (v) the effect of post-storage drying methods do not affect the DNA content in leaf tissue during storage drying interaction; and (vi) the effect of temperature treatments is the same for different leaf tissues (leaf tissue and post-storage drying interaction).

RESULTS AND DISCUSSION

DNA Content in Leaf Tissue during Storage

In a preliminary experiment, we pur-
chased and tested several commercially available plant designated DNA extraction kits to determine their efficiency, consistency, and repeatability in extracting the highest content and quality of genomic DNA from fresh, air-dried, and oven-dried leaf tissue of *E. c. var. coca* Lam. (unpublished data). The most effective and reliable plant DNA extraction kit was the DNeasy Plant Mini kit from Qiagen. We also observed that the content of DNA extracted from freshly harvested rolled and mature leaf tissue of *E. c. var. coca* differed by 4-fold (unpublished data); therefore, both leaf types were used in the current study. This procedure would ensure that the DNA content and quality of both rolled and mature, as well as air- and oven-dried *E. c. var. coca* leaf tissue, would be known. All subsequent DNA data and results reported in the current study were obtained with the DNeasy kit.

Figure 1, A–D, shows the data for rolled and mature leaf tissue of *E. c. var. coca* that were harvested and stored for 50 days. As previously observed, there was a 4-fold difference between the content of DNA in freshly harvested, rolled, and mature coca leaf tissue, which may be attributed to leaf age. From day 0 to day 10 of storage, there was no significant change in DNA content in rolled leaf tissue stored at -20° and 23°C; however, DNA in rolled leaf tissue stored at 4°C on day 10 was significantly different (Figure 1A). We attribute this difference to DNA condensation, which is also prevalent in mature leaf tissue on day 10 (Figure 1A). We attribute this difference to DNA condensation, which is also prevalent in mature leaf tissue on day 10 (Figure 1A). Rolled leaf tissue stored at 23°C had declined to 0.74 µg/50 mg dry weight, a 74% loss (Figure 1A).

The DNA content in mature leaf tissue of *E. c. var. coca* stored at 4°C, from day 0 to day 10, increased from 0.69 µg/50 mg dry weight leaf tissue to 1 µg/50 mg dry weight leaf tissue and remained static up to day 20 (Figure 1B). After day 20 of storage, DNA content in mature leaf tissue (4°C) decreased to a low of 0.52 µg/50 mg dry weight leaf tissue (day 30), increasing again to 0.77 µg/50 mg dry weight leaf tissue at day 50 (Figure 1B), exhibiting essentially no loss in DNA content from day 0. DNA

Table 2. AFLP Analyses Using DNA Leaf Extracts of Rolled and Mature *E. c. var. coca* Leaf for 0, 10, and 50 Days

<table>
<thead>
<tr>
<th></th>
<th>Rolled</th>
<th>Mature</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>4°C</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>23°C</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>-20°C</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

A + sign indicates successful amplified fragment-length polymorphism (AFLP) and fragment analysis. Only 4°, 23°, and -20°C samples were analyzed, as air and oven drying proved impractical for maintaining DNA integrity. Although the 23°C samples stored for 50 days showed extensive degradation, DNA integrity was sufficient for AFLP analysis. However, the quality of the fragment data was sub-par compared with the 4° and -20°C samples.

Figure 1. Storage effects on genomic DNA integrity in rolled and mature *E. c. var. coca* leaf tissue extracts. DNA integrity as a function of storage parameters over 50 days in (A) rolled, (B) mature, (C) rolled oven-/air-dried, and (D) mature oven-/air-dried.
content in mature E. c. var. coca leaf tissue stored at -20°C declined to a low of 0.54 µg/50 mg dry weight leaf tissue at day 30, increased to 0.74 µg/50 mg dry weight leaf tissue at day 40 and remained static up to day 50 of storage, again resulting in no loss in DNA content (Figure 1B). By day 20 of storage mature E. c. var. coca leaf tissue stored at 23°C declined to a low of 0.32 µg/50 mg dry weight leaf tissue and remained static through day 50, showing a 49% loss in DNA content (Figure 1B). Figure 2 depicts the quality of extracted DNA in both rolled and mature E. c. var. coca leaf tissue at 0, 10, and 50 days of storage for 4°, -20°, and 23°C treatments. DNA qualitative results (Figure 1, A and B) mimicked the quantitative results for both rolled and mature leaf tissue, in that 4° and -20°C samples showed minimal degradation over 50 days, while 23°C exhibited some breakdown after 10 days and extensive degradation after 50 days (Figure 2). However, quantitative results are not always indicative of DNA quality.

The effect of air (23°C) and oven drying (40°C) on DNA content are shown in Figures 1 and 2. Through the first 10 days of storage DNA content in rolled, oven-dried, and air-dried leaf tissue declined to 1.3 (41% loss) and 0.92 µg/50 mg dry weight leaf tissue (36% loss), respectively (Figure 1C). Both air- and oven-dried tissue continued a gradual decline in DNA content where at day 50 contents were 0.23 (84% loss) and 0.35 µg/50 mg dry weight leaf tissue (84% loss), respectively. By day 10 of storage in mature, air-dried, and oven-dried leaf tissue, DNA content was 0.26 and 0.27 µg/50 mg dry weight leaf tissue and by day 50 had declined to 0.20 and 0.14 µg/50 mg dry weight leaf tissue, a 70% and 75% loss, respectively (Figure 1D). Quality of DNA was also sacrificed by the drying methods (Figure 2). While 50-day rolled and mature dried samples exhibited less DNA quantitatively than 0-day samples, qualitatively there was little DNA degradation over 50 days of storage, beyond what was degraded during the initial drying process. We do not recommend the use of air or oven drying for E. c. var. coca leaf tissue for DNA studies because the time required for degradation is ephemeral and the amount and quality of DNA extracted are less than desired (Figure 1, C and D, and Figure 2).

DNA from rolled and mature leaf tissue stored for 0, 10, and 50 days was tested for integrity by AFLP analysis (see Materials and Methods section) with the five primer sets listed in Table 1. Table 2 shows the results of the AFLP analyses of DNA from rolled and mature leaf tissue stored for 0, 10, and 50 days. It is noteworthy that the quality of the DNA from all stored tissue types tested was sufficient for AFLP analyses.

We attribute the differences observed in DNA quality and quantity within E. c. var. coca leaf tissue to the conditions under which the leaf tissue was treated (Figures 1 and 2). Rolled leaf tissue (4° and -20°C) maintained the highest DNA content and quality throughout the study, while all treatments of mature leaf tissue of E. c. var. coca had lower quantities of DNA before and during storage (Figures 1 and 2). The increase observed in DNA content between day 0 and day 10 in rolled and mature leaf tissue stored at 4°C is probably due to condensation as suggested by several researchers (E.L.J., personal communications). They summarized that it was not uncommon to observe an increase in DNA content in leaf tissue over the first several days of storage (E.L.J., personal communication), and their suggestions apparently are merited because there was not an increase in DNA content of this magnitude during the duration of the study (Figure 1, A–D).

For the current research, we did not label each leaf type from bud break to maturity, nor did we use leaf tissue from individual plants of the taxon, which would have been more definitive for DNA in terms of content and interspecific variation. This technique may have circumvented some of the variations that are known to exist within the taxon. Our desire was to mimic harvest of the two E. c. var. coca leaf

<table>
<thead>
<tr>
<th>Orthogonal Contrast</th>
<th>Day 10</th>
<th>Day 50</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Rolled vs. mature</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td>2. Dry vs. no-dry at 23°C</td>
<td>***</td>
<td>*</td>
</tr>
<tr>
<td>3. Low temperature vs. 23°C</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td>4. Air dry vs. oven dry</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>5. -20° vs. 4°C</td>
<td>***</td>
<td>*</td>
</tr>
<tr>
<td>6. Interaction (1 * 2)</td>
<td>***</td>
<td>ns</td>
</tr>
<tr>
<td>7. Interaction (1 * 3)</td>
<td>ns</td>
<td>***</td>
</tr>
</tbody>
</table>

*Significant at the 0.05 level
***Significant at the 0.001 level

**Table 3. Orthogonal Contrasts Testing Hypotheses of Interest**

**Figure 2.** Gel electrophoresis (1% agarose gel) of genomic DNA (150 ng/lane) from rolled and mature leaf tissue extracts of E. c. var. coca harvested and stored in RNALater for 0, 10, and 50 days. Samples are as follows: lanes 1, 6, 11, 16, 21, and 26, 4°C samples; lanes 2, 7, 12, 17, 22, and 27, -20°C samples; lanes 3, 8, 13, 18, 23, and 28, 23°C samples; lanes 4, 9, 14, 19, 24, and 29, oven-dried samples; and lanes 5, 10, 15, 20, 25, and 30, air-dried samples. d, days.
tissue types that would occur if samples were collected under field conditions so that DNA obtained would thus be reflective of the storage. We have designed future research that will address these differences.

Statistical Analyses

The analyses breaking down the effects of storage temperature and post-storage drying have been performed using orthogonal contrasts (Table 3). The orthogonal contrast at 10 and 50 days of storage revealed that the hypothesis that rolled and mature leaf tissue contained the same DNA content should be rejected, verifying that rolled leaves contained higher DNA content than mature leaves (contrast 1). Post-storage drying decreased DNA content on both days of storage (contrast 2). Low-temperature (-20° and 4°C) storage resulted in higher DNA content than room temperature (23°C) at both days of storage (contrast 3). There was no difference in DNA content between air- and oven-dried samples at either of the storage days (contrast 4). Storage at 4°C resulted in higher DNA content than -20°C at 10 days of storage, whereas no difference was found at 50 days of storage (contrast 5). Reduction in DNA content due to post-storage drying was more pronounced for rolled leaves than mature leaves at 10 days of storage \( (P < 0.01) \), while no such interaction was detected at 50 days of storage \( (P = 0.334) \) (contrast 6). There was no interaction between leaf age and low-temperature storage at 10 days of storage \( (P = 0.321) \). However, storing at low temperature preserved DNA markedly more for rolled leaves than mature leaves at 50 days of storage \( (P < 0.001) \) (contrast 7).

CONCLUSION

This research demonstrates how the content of DNA differs within two developmental stages of *E. c. var. coca* leaf tissue, in terms of storage and preservation in RNA later. Higher content and quality DNA was present in rolled leaf tissue that persisted from the first harvest (day 0) to 50 days of storage. The ideal temperature for storage of *E. c. var. coca* leaf tissue that will maintain high-quality DNA and content for 50 days was found to be 4°C and -20°C. However, rolled leaf tissue of *E. c. var. coca* stored at 23°C will also provide DNA with sufficient content and quality when stored for 10 days or less. Therefore, the content and quality of DNA in rolled leaves of *E. c. var. coca* harvested in tropical regions and immediately stored in RNA later should not be affected for the first 10 days of storage. The major advantages of this method for storing *E. c. var. coca* leaf tissue for DNA studies is (i) it is convenient and portable; (ii) it may be stored without refrigeration up to 10 days after harvest; (iii) it can be stored up to 50 days at 4°C or -20°C while maintaining high-quality DNA and RNA; (iv) it is cost-effective in terms of leaf tissue transport and equipment; and (v) it maintains the quality of the DNA sufficiently enough (and without PCR inhibitors) to run successful AFLP analyses.

ACKNOWLEDGMENTS

Commercial products are listed for the reader’s convenience only and indicate neither endorsement by the U.S. Department of Agriculture Research Service, nor criticism of similar products not mentioned.

REFERENCES


Received 2 December 2002; accepted 14 April 2003.

Address correspondence to Emmanuel L. Johnson, USDA ARS Alternate Crops and Systems Laboratory, Bldg. 001 Rm. 329 BARC-W, 10300 Baltimore Ave., Beltsville, MD 20705-2350, USA. e-mail: johnson.e@ba.ars.usda.gov