A Simple and Efficient Method for Isolation of DNA in High Mucilaginous Plant Tissues

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Abstract

A protocol is described for rapid DNA isolation from Malvaceae plant species and different tissues of Bixaceae that contain large amounts of polysaccharides, polyphenols, and pigments that interfere with DNA extractions. The method is a modification of Dellaporta et al. The current protocol is simple, and no phenol-chloroform extraction, ethanol, or isopropranol precipitation is required. The method is based in the incubation of soluble DNA with silica, mix in batch during the extraction. The procedure can be completed in 2 h and many samples can be processed at the same time. DNA of excellent quality was recovered and used for polymerase chain reaction (PCR) amplification, restriction enzyme digestion, and Southern blot analysis. The method was used with healthy *Bixa orellana* and virus-infected Malvaceae plants.

Index Entries: DNA extraction; Malvaceae; PCR; *Bixa orellana*.

1. Introduction

The extraction of high-quality DNA is an important step in many molecular biology protocols, and several factors alter the amount, purity, and quality of the extracted molecules. The method described is a modification of the one by Dellaporta et al. ***(1)***. Plant species of the Malvaceae family are characteristically high in polysaccharides and other secondary metabolites, which interfere with effective DNA extractions and polymerase chain reaction (PCR) amplification. Polysaccharides have viscous, glue-like texture and make the DNA unmanageable in pipetting and unsuitable for PCR because polysaccharides inhibit *Taq* polymerase activity ***(2)***. Many protocols for DNA extraction from Malvaceae plant species rich in polysaccharides, polyphenols, and latex have been described. However, these protocols are generally time consuming or expensive ***(3)***. Bayer et al. ***(4)*** reported that many Malvaceae samples contain high amounts of mucilage, and it is difficult to remove the mucilaginous supernatant after centrifugation without losing most of the DNA. Jose and Usha (***5***) described a protocol for the extraction of geminiviral DNA from *Abelmoscus esculentus*, a Malvaceae plant. This method involves a combination of Dellaporta et al. ***(1)***, the CTAB (cetyltrimethylammonium bromide) method ***(6)***, and alkali lysis. Other techniques for DNA extraction from different highly mucilaginous plants, whichh include modifications of the CTAB-based protocols, have been reported ***(7–9)***.

Similar problems in DNA extraction occurs with *Bixa orellana*, the only species within the family Bixaceae. This plant produces a variety of secondary metabolites in all of its tissues. The high content of phenols and polysaccharides represents a problem in the extraction of high-quality DNA.

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Molecular studies in *B. orellana* are very scarce despite its economical value because of the large amounts of bixin found in the seeds. This apocarotenoid is widely used as a colorant in the food industry ***(10)***. In this case, the quality of the DNA isolated is a critical step in molecular and genetic studies. Narváez et al. ***(11)*** used a methodology for genomic DNA isolation from *B. orellana* tissues, previously reported by Rogers and Bendich ***(12)***. This method is based on the CTAB extraction procedure. This technique uses extraction with chloroform/isoamyl alcohol, precipitation with ethanol, and others steps that are time consuming. Also, a CTAB extraction procedure does not guarantee the elimination of some contaminants of DNA such as polysaccharides and lipids. When DNA from *B. orellana* was extracted following Rogers and Bendich's method ***(12)***, a wide variation in the yield of nucleic acids was obtained, as a result of the interference by their high polysaccharide content.

Despite isolation of specific probe for analyzing the expression of mRNAs of enzymes related with isoprenoid biosynthesis in *B. orellana* ***(13)***, the isolation of complete genes has been very difficult in this species using plant DNA extracted by reported methods. This is possibly because of the presence of a contaminant in DNA extract that interferes with the gene isolation process.

The protocol reported here produces clean high molecular weight genomic DNA from fresh or herbarium material of Malvaceae plant species that presented viral symptoms, without phenol, ethanol precipitation, or cesium chloride gradient. Also, the method yields DNA extract from different healthy tissues of *B. orellana,* without the viscous appearance that indicates the presence of a high polysaccharide content. Both DNA extracts from *B. orellana* tissues and Malvaceae species were used in analytical applications, such as restriction digestion, Southern blot analysis, and PCR, producing a good restriction pattern and amplification, as indicated by stain with ethidium bromide.

2. Materials

2.1. Plant Material

Malvaceae plant species with viral symptoms were collected in different locations in the Yucatan Peninsula, stored on ice after collection, and stored at –80°C until used. Dry herbarium material of Malvaceae species were collected from plants that showed possible viral symptoms from the Herbarium CICY. Three leaves of each herbarium sample were taken and stored at –80°C until DNA was extracted. Different tissues of adult plants of *B. orellana* L. (Annatto) var. Criolla such as leaf, flower buds, open flowers, fruits, and seed were collected during the flowering and fruiting period (September through October) at "La Extra" plantation located in Chicxulub Pueblo, Yucatán, México. Fresh tissues were immediately frozen in liquid nitrogen and kept at –80°C for later use.

2.2. Equipment and Reagents

1. Mortar and pestle.
2. 2.0-mL eppendorf tubes autoclaved.
3. Extraction buffer: 10 m*M* Tris-HCl, 50 m*M* EDTA (ethylendiaminetetraacetic acid), 500 m*M* NaCl (sodium chloride), 10 m*M* β-mercaptoethanol, pH 7.0.
4. 20% sodium dodecyl sulfate (SDS).
5. 5 *M* potassium acetate.
6. Silica (Sigma S5631). Resuspended 2 g of silica in 15 mL of distilled water and spun at 756*g* for 1 min. Eliminated the milky supernatant. Repeated twice and resuspended silica in 50 mL of distilled water. Solution could be stored at room temperature.
7. Red*Taq* polymerase (Bioline).
8. Restriction enzymes (Invitrogene).
9. dNTPs mix (Bioline).
10. PCR thermal cycler (96-well plate format).

3. Methods

3.1. DNA Extraction

The method developed by Dellaporta et al. ***(1)*** was modified to recover DNA from leaves of Malvaceae plant species: *Corchorus siliquosus*,

Malvastrum coromandelianum, *Abutilon permolle*, *Hibiscus sabdariffa*, *Sida acuta*, *Herissantia crispa*, *Anoda cristata*, *Sida rhombifolia*, *Bastardia viscosa*, and Bixaceae spp., *B. orellana*.

1. Homogenized 0.1 g of leaf material or others tissues with 1 mL of extraction buffer, transferred to a 2.0-mL eppendorf tube, and added 100 µL of 20% SDS. After mixing, incubated at 65°C for 10 min.
2. Added 500 µL of 5 *M* potassium acetate. Shook tube vigorously and incubated for 20 min on ice.
3. Spun tubes at 16,000*g* for 20 min.
4. Transferred supernatant to a new 1.5-mL tube and add 300 µL of silica, mixed manually for 3–5 min.
5. Spun tubes at 16,000*g* for 30 s.
6. Washed pellet twice with 70% ethanol. Dried the pellet.
7. Resuspended the pellet in 50 µL of distilled water and incubated at 55°C for 5 min.
8. Spun tubes at 16,000*g* for 2 min and transferred supernatant to a new 500-µL eppendorf tube.
9. Used an aliquot for quantification of total DNA in gel electrophoresis with 1% agarose.

3.2. DNA Analysis

3.2.1. Polymerase Chain Reaction

Genomic DNA from Malvaceae species: 50 ng of template DNA extract were added to a final volume of 25 µL containing a previously standardized PCR master mix. Core Coat Protein fragments of begomoviruses were obtained by PCR amplification, using 20 pmol of degenerate primers AV324 and AC1154, capable of universal amplification of most begomoviruses ***(14)***. The reaction mixture was run on a thermal cycler as follow: 94°C for 5 min, 35 cycles of 95°C for 1 min, 58°C for 1 min, and 72°C for 1 min, and one final cycle at 72°C for 10 min, 25°C for 20 min, and 4°C infinite.

The genomic DNA from *B. orellana* leaf tissue was analyzed for the presence of genes involved in the isoprenoids biosynthesis using specific oligonucleotide primers. The reaction mixture was run on a thermal cycler as follow: 94°C for 5 min, 35 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min, and one final cycle at 72°C for 10 min, 25°C for 20 min, and 4°C infinite.

The amplified products were assayed by electrophoresis on 1% agarose gels and stained with ethidium bromide.

3.2.2. Restriction Analysis

Ten DNA samples of different Malvaceae species were digested by overnight restriction with *Eco*RI. The susceptibility of genomic DNA from *B. orellana* leaf tissue to cleavage by *Eco*RV, *Bam*HI, *Hind*III, *Xba* I, *Sac* I, and *Sau* 3A was determined by overnight restriction with these enzymes.

3.2.3. Southern Blot Analysis (see ***Note 5***)

Four hundred nanograms of total DNA from different Malvaceae species (*Corchorus siliquosus*, *Malvastrum coromandelianum*, *Abutilon permolle*, *Hibiscus sabdariffa*, *Sida acuta*, *Herissantia crispa*), were electroforesed in 1% agarose gel (without ethidium bromide), and transferred to Hybond (+) nylon membranes (Amersham, Arlington Heights), by standard protocols. Hybridization reaction were performed with a Alkaline phosphatase labeled (Alkphos Direct Hibridization kit, Amersham) core Coat protein probe of the *Pepper golden mosaic virus* (576 nt fragment), as recommended by the supplier.

4. Notes

1. In the protocol reported here, no phenol–chloroform, ethanol, or isopropanol precipitation were used, and we obtained clean high molecular weight genomic DNA from fresh healthy and viral-infected tissue and from herbarium material (**Fig. 1**). This method is inexpensive, easy, and quick. Herbarium material of Malvaceae plants contains greater amounts of polyphenols than fresh material, but with this protocol DNA of excellent quality (**Fig. 1A**) is obtained.

   The total genomic DNA isolated by this method provided intact DNA in all assayed tissues of *B. orellana* (**Fig. 1B**). The mucilaginous texture observed in DNA extracts obtained

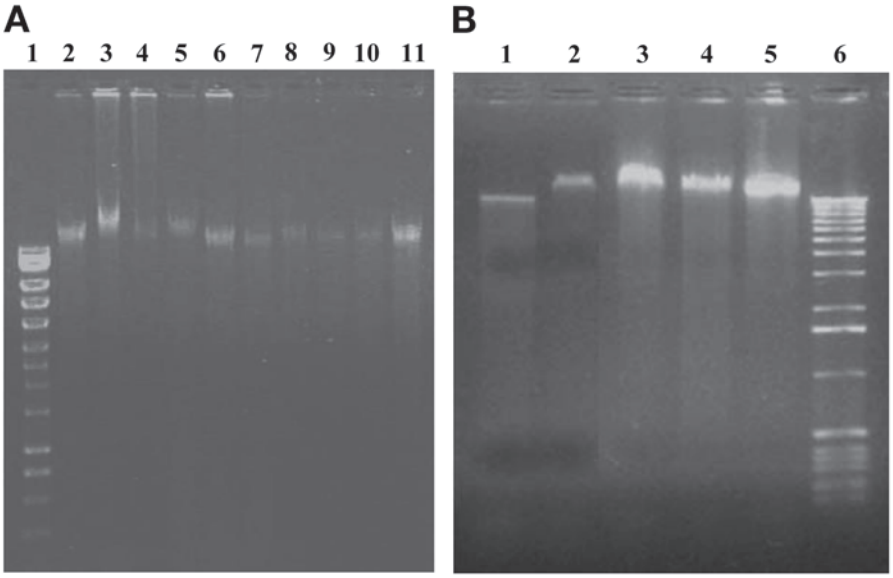

Fig. 1. Agarose gel electrophoresis of DNA extraction. **(A)** ADN extract from Malvaceae species. Lanes: 1: MWM (Hyper ladder I, Bioline); 2: *Corchorus siliquosus;* 3: *Malvastrum coromandelianum;* 4: *Abutilon permolle;* 5: *Hibiscus sabdariffa;* 6: *Sida acuta;* 7: *Herissantia crispa;* 8: *Anoda cristata;* 9: *Sida rhombifolia;* 10: *Abutilon permolle;* 11: *Bastardia viscosa*. Lanes 2–9 were obtained from fresh material and lanes 10 and 11 were obtained from herbarium material. **(B)** ADN extracts from different tissues of *Bixa orellana*. Lanes: 1: seed; 2: flower; 3: flower bud; 4: fruit; 5: leaf; 6: MWM (marker X, 0.07–12.2 Kbp, Roche).

Table 1
DNA Yields Obtained Using Different Amounts of Plant Tissue

Tissue (fresh weight, g)	0.1	0.2	0.3	0.4	0.5	0.6
Bixa orellana DNA (μg/μL)	0.2	0.32	0.8	0.65	0.6	0.6
Malvastrum coromandelianum DNA (μg/μL)	0.2	0.35	0.48	0.6	0.84	0.96

The DNA extractions were done using healthy *Malvastrum coromandelianum* and *Bixa orellana* plants.

using others methodologies ***(1,6,12,15,16)***, especially from flower buds, open flowers, and fruits tissues extracts in adult plants, disappeared with this method.

The amount of total DNA obtained from 0.1 g of fresh tissue by various extractions wase determined spectofotometrically. The concentration of total DNA of *M. coromandelianum and B. orellana* is shown in **Table 1**. The concentration of DNA of *M. coromandelianum* increased linearly as the amount of tissue increased, but in *B. orellana*, the higher amount of DNA was obtained with 0.3 g of fresh leaf tissue (**Table 1**).

A second silica elution is recommended to increase the efficiency of the extraction.

2. An additional wash with 70% ethanol was necessary to eliminate the pigment in the flower buds and seed extracts from *B. orellana*. The presence of RNA in the extracts was totally eliminated by adjusting the distilled water to a pH above 7.0, without RNase treatment.
3. We were able to successfully amplify begomoviruses DNA fragments (840 bp of coat protein gene) using DNA from all the extractions of the different Malvaceae samples, from fresh tissue and from herbarium material **(Fig. 2A**).

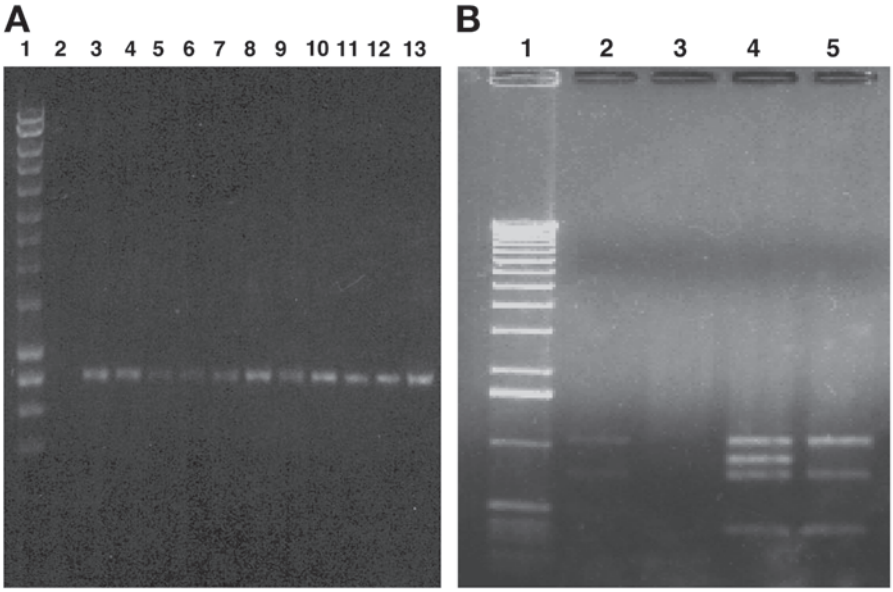

Fig. 2. Agarose gel electrophoresis of PCR products. **(A)** PCR products for Malvaceae species. Lanes: 1: MWM (Hyper ladder I, Bioline); 2: negative control (without genomic DNA extract); 3: *Corchorus siliquosus;* 4: *Malvastrum coromandelianum;* 5: *Abutilon permolle;* 6: *Hibiscus sabdariffa;* 7: *Sida acuta;* 8: *Herissantia crispa;* 9: *Anoda cristata;* 10: *Sida rhombifolia;* 11: *Abutilon permolle;* 12: *Bastardia viscosa;* 13: positive control (*Pepper golden mosaic virus* DNA A). Lanes 3–10 were obtained from fresh material and lanes 11 and 12 were obtained from herbarium material. **(B)** The PCR products for *Bixa orellana*. Lanes: 1: MWM (marker X, 0.07–12.2 Kbp, Roche); 2: positive control (partial HMGR genes from *Bixa* using degenerate primer) ***(12)***; 3: negative control (without genomic DNA extract); 4 and 5: PCR products using two different combinations of oligonucleotide primers.

We used 20–50 ng of DNA in all samples. Two DNA samples extracted from herbarium material presented possible viral symptoms, like mosaic, were positive to begomoviruses amplification.

The PCR analysis of genes involved in the isoprenoids biosynthesis in the genomic DNA from *B. orellana* leaf tissue, resulted in good amplification, corresponding to expected size ***(13)***, as indicated by staining with ethidium bromide (**Fig. 2B**). The amplification by PCR using the same oligonucleotide primers has been impossible to obtain using as template DNA extracted by different assays ***(1,12,16)***. This result is indicative of DNA free of polysaccharide and other contaminants that are particularly problematic, affecting the PCR by inhibiting *Taq* DNA polymerase activity or some restriction enzymes.

4. The DNA obtained can be digested by restriction enzymes. We used the *Eco*RI restriction enzyme for genomic DNA from Malvaceae species and the results are shown in **Fig. 3A**. All the samples analyzed were digested with the restriction enzyme. Also, genomic DNA of *B. orellana* leaf tissue was susceptible to cleavage by restriction enzymes such as *Eco*RV, *Bam*HI, *Hind*III, *Xba*I, *Sac*I, and *Sau*3A (**Fig 3B**).
5. Southern hybridization analysis was conducted using total DNA extracted from 0.1 g of different Malvaceae species that presented viral symptoms. The probe used was a core CP fragment obtained from amplification of PepGMV DNA A cloned in the pGEM 7z (+). Results revealed that each DNA sample was capable of producing open circle (OC), single strand (SS), and double strand DNA (DS) forms of the viral genome (**Fig. 4**). All of the DNA samples ana-

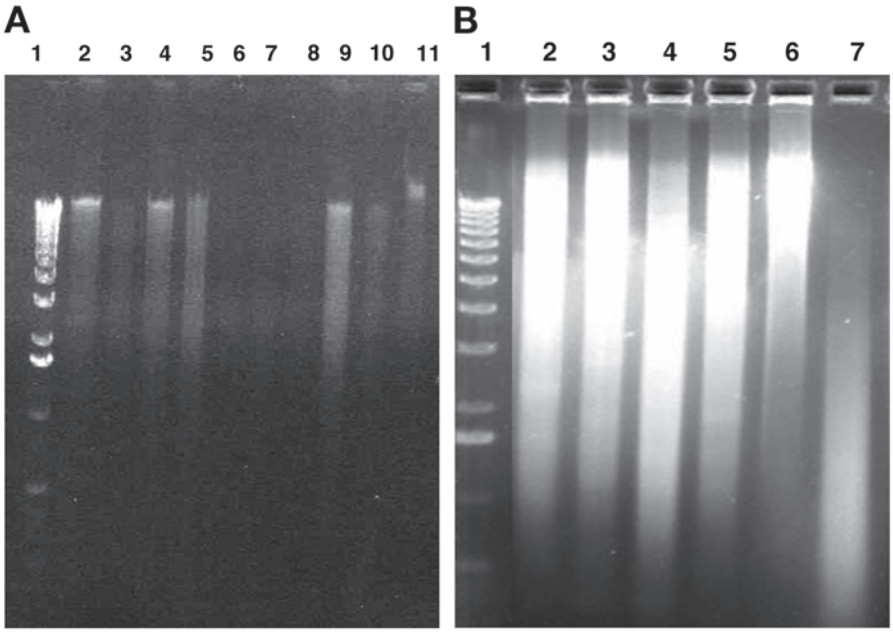

Fig. 3. Restriction analysis of total genomic DNA. **(A)** Malvaceae plants. Lanes: 1: MWM (Hyper ladder I, Bioline); 2: *Corchorus siliquosus;* 3: *Malvastrum coromandelianum;* 4: *Abutilon permolle;* 5: *Hibiscus sabdariffa;* 6: *Sida acuta;* 7: *Herissantia crispa;* 8: *Anoda cristata;* 9: *Sida rhombifolia;* 10: *Abutilon permolle;* 11: *Bastardia viscosa*. Lanes 2–9 were obtained from fresh material and lanes 10 and 11 were obtained from herbarium material. *Eco*RI was used for DNA digestion. **(B)** *Bixa orellana* leaf tissue. Lanes: 1: MWM (Hyper ladder I, Bioline); 2: *Eco*RV; 3: *Bam*HI; 4: *Hind*III; 5: *Xba*I; 6: *Sac*I; 7: *Sau*3A.

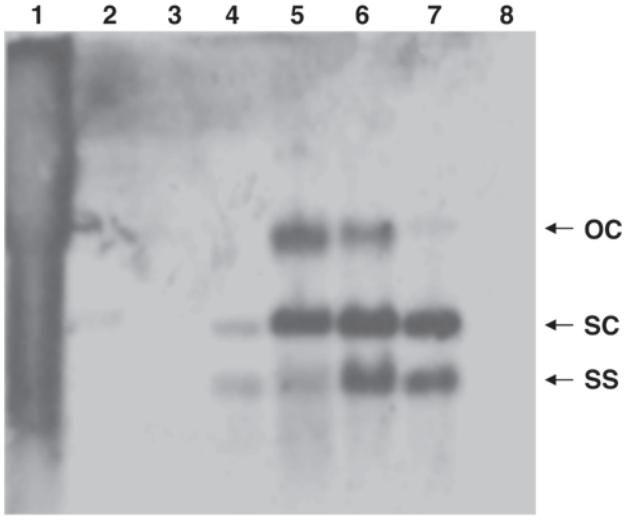

Fig. 4. Southern blot analysis of Malvaceae species. Lanes: 1: positive control (*Pepper golden mosaic virus* DNA A); 2: *Corchorus siliquosus;* 3: *Malvastrum coromandelianum;* 4: *Abutilon permolle;* 5: *Hibiscus sabdariffa;* 6: *Sida acuta;* 7: *Herissantia crispa;* 8: negative control. Using total DNA, 400 ng of each sample was used for the analysis. The mobilities of single-stranded (SS), supercoiled (SC), and open circular (OC) forms of the standard viral genome are indicated at right. Hybridization was conducted using a Alkphos (Amersham)-labeled probe of a fragment of the coat protein (core CP) of *Pepper golden mosaic virus* DNA A.

lyzed were detected by Southern blot analysis using the silica method.

This method may be useful for other plant species containing high levels of polysaccharides and polyphenols. We used it for DNA extraction of Cactaceae plants, with excellent results for PCR amplification (data not shown). The average execution time for this protocol was approx 2 h, handling 12 samples at the same time.

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References

1. Dellaporta, L., Wood, J., and Hicks, J. B. (1983) A plant DNA minipreparation: version II. *Plant Mol. Biol. Rep.* **1,** 19–21.
2. Fang, G., Hammar, S., and Grumet, R. (1992) A quick and inexpensive method for removing polysaccharides from plant genomic DNA. *Biofeedback* **13,** 52, 53.
3. Jeske, H., Lutgemeier, M., and Preib, W. (2001) DNA forms indicate rolling circle and recombination-dependent replication of *Abutilon mosaic virus*. *EMBO J.* **20,** 6158–6167.
4. Bayer, C., Fay, M. F., De Bruijn, A. Y., et al. (1999) Support for an expanded family concept of Malvaceae within a recircumscribed order Malvales: a combined analysis of plastid *atpB* and *rbcL* DNA sequences. *Bot. J. of Linnean Soc.* **129,** 267–303.
5. Jose, J. and Usha, R. (2000) Extraction of geminiviral DNA from a highly mucilaginous plant (*Abelmoscus esculentus*). *Plant Mol. Biol. Rep.* **18,** 349–355.
6. Doyle, J. J. and Doyle, L. J. (1990) Isolation of plant DNA from fresh tissue. *Focus* **12,** 13–15.
7. Mansoor, S., Khan, S. H., Bashir, A., et al. (1999) Identification of a novel circular single-stranded DNA associated with cotton leaf curl disease in Pakistan. *Virology* **259,** 190–199.
8. Barnwell, P., Blanchard, A. N., Bryant, J. A., Smirnoff, N., and Weir, F. (1998) Isolation of DNA from the highly mucilaginous succulent plant *Sedum telephium*. *Plant Mol. Biol. Rep.* **16,** 133–138.
9. Scott, K. D. and Playford, J. (1996) DNA extraction technique for PCR in Rain Forest plant species. *Biotechniques* **20,** 974–978.
10. Aparnathi, K., Lata, R., and Sharma, R. (1990) Annatho (*Bixa orellana* L.): its cultivation preparation and usage. *Intern. J. Trop. Agric.* **8,** 80–88.
11. Narváez, J. A., Flores-Pérez, P., Herrera-Valencia, V., et al. (2001) Development of molecular techniques for studying the metabolism of carotenoids in *Bixa orellana* L. *Hortscience* **36,** 982–986.
12. Rogers, S. O. and Bendich A. J. (1985) Extraction of DNA from milligram amounts of fresh, herbarium and mummified plant tissues. *Plant Mol. Biol.* **5,** 69–76.
13. Narváez, J. A., Canto-Canché, B. B., Flores-Pérez, P., and Rivera-Madrid, R. (2001) Differential expression of 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) during flower and fruit development of *Bixa orellana*. *J. Plant Physiol.* **158,** 1471–1477.
14. Idris, A. M. and Brown, J. K. (1998) Sinaloa tomato leaf curl geminivirus: biological and molecular evidence for a new subgroup III virus. *Phytopathology* **88,** 648–657.
15. Porebski, S., Bailey, G., and Baum, B. R. (1997) Modification of CTAB DNA extraction protocol for plants containing high polysaccharide and polyphenol components. *Plant Mol. Biol. Rep.* **15,** 8–15.
16. Lodhi, M. A., Ye, G. N., Weeden, N. F., and Reisch, B. I. (1994) A simple and efficient method for DNA extractions from grapevine cultivars and *vitis* species. *Plant. Mol. Biol. Rep.* **12,** 6–13.