CHAPTER 4

EXTRACTION OF GENOMIC DNA FROM ROOTS AND LEAVES OF *H. undatus*

4.1 INTRODUCTION

Most of the DNA extraction protocols extract high quality DNA using young leaves as starting materials (Lodhi *et al.*, 1994, Havania *et al.*, 2004, and Couch and Fritz, 1990). However due to the characteristic of *Hylocereus undatus*, leaves which are modified to needle like structure could not provide a large number of cells per unit volume resulting in low concentration of nuclei. Therefore, leaves are not suitable for DNA extraction for *Hylocereus undatus*.

Tel-Zur *et al.*, 1999, used roots (Fig 4.1) as starting material because the tissue source has lower viscosity of extracts as compared to other tissues such as stems (Tel-Zur *et al.*, 1999). Diadema *et al.*, 2003, used callus as starting material to obtain an average DNA yield of 1.8mg/g. However, generating callus requires time, space, expertise in tissue culture and most importantly is highly costly. Usage of leaf callus as a source of material is due to absence of the differentiation in callus preventing the synthesis of secondary metabolites (Diadema *et al.*, 2003).

The aim of this chapter is to establish an efficient DNA extraction used seedling leaves material (Fig 4.2) generated during germination as the starting material and compared to using roots. Young leaf material was preferred because it is relatively soft making it easily homogenised as well as containing high number of actively dividing cells. The

modification done in this DNA extraction was the addition of Sorbitol, Sodium Bis-sulphite and Sodium Sarkosyl to the extraction buffer. Sodium Bis-sulphite and Sodium Sarkosyl prevent the oxidation of phenolic compounds and precipitate high levels of polysaccharides respectively (Havania *et al.*, 2004 and Sharma *et al.*, 2002). Antioxidants are commonly used to overcome problems related with phenolics. Phenolics interfere with the extracted DNA making it difficult to separate the DNA. β-mercaptoethanol and Polyvinylpyrrolidone were used as antioxidants during the DNA extraction. EDTA was used to chelate magnesium ions, a co-factor for nucleases which degrades the released DNA.

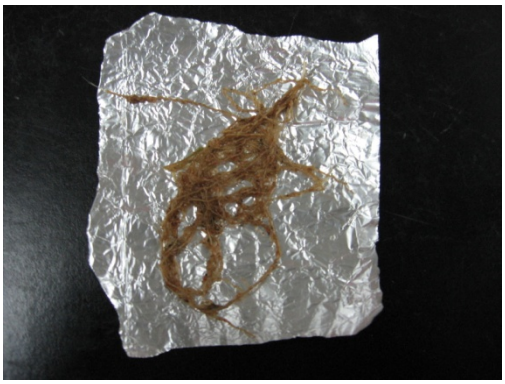

Fig 4.1: Roots for DNA extraction

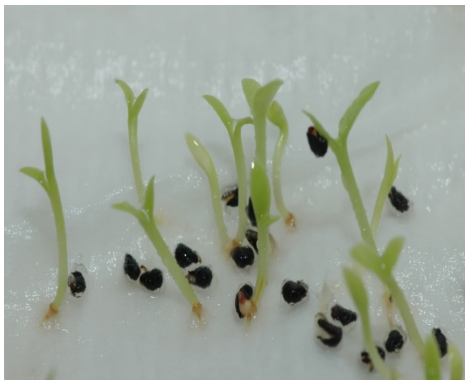

Fig 4.2: Young leaflets for DNA extraction

4.2 MATERIALS AND METHODS

4.2.1 Plant Material

Plant material was obtained as described in Section 3.2 and the roots were taken from *H.undatus* plants grown outdoor.

4.2.2 Preparation of Reagents

4.2.2.1 DNA isolation buffer (Modified method)

a) 1.37g of Sorbitol (Sigma)
b) 0.76g of Tris-base (Sigma)
c) 0.73g of EDTA (BDH)
d) 2.43g of Sodium Chloride (Sigma)
e) 0.42g of Hexadecyltrimethyl-ammonium bromide (CTAB) (Sigma)
f) 0.42ml of Sodium Sarkosyl (BioWhittaker)
(a) - (f) were dissolved in 50ml of sterile distilled water, autoclaved and stored at room temperature. 0.5g of Polyvinylpyrrolidone (PVP) (MW 40,000) (Research Organic) and 100µl of β-mercaptoethanol (AppliChem) and 57mg of Sodium Bis-sulphite (R&M) were added fresh before DNA extraction.

4.2.2.2 Extraction buffer (Tel-Zul *et., al* 1999)

a) 12.76g of Sorbitol (Sigma)
b) 100ml of (200mM Tris-HCl, pH 8.0)
c) 2ml of (0.5M EDTA, pH 8.0)
(a)-(c) were added to 50ml of sterile distilled water and volume made up to 200ml with sterile distilled water. The solution was autoclaved and stored at room temperature. 2 ml of β-mercaptoethanol (AppliChem) was added fresh before use.

4.2.2.3 High-salt CTAB buffer (Tel-Zul *et., al* 1999)

a) 11.69g of Sodium Chloride (Sigma)
b) 0.9g of Hexadecyltrimethyl-ammonium bromide (CTAB) (Sigma)
(c) 5ml of (200mM Tris-HCl, pH 8.0)
(d) 2.5ml of (0.5M EDTA, pH 8.0)
(a)-(d) were to 25ml of sterile distilled water and volume made up to 50ml with sterile distilled water. The solution was autoclaved and stored at room temperature.

0.5M EDTA

14.61g of EDTA (BDH) was dissolved in sterile distilled water; pH was adjusted to 8.0 with Sodium hydroxide pellets (Merck) and volume made up to 100ml with sterile distilled water. The solution was autoclaved and stored at room temperature.

200mM Tris-HCl

12.12g of Tris-base (Sigma) was dissolved in sterile distilled water; pH was adjusted to 8.0 with Sodium hydroxide pellets (Merck) and volume made up to 500ml with sterile distilled water. The solution was autoclaved and stored at room temperature.

Chloroform : isoamyl alcohol (24:1)

2ml of Isoamyl alcohol (R&M) was added to 48ml of Chloroform (R&M). The solution was stored at room temperature.

5M Sodium Chloride

14.61g of Sodium Chloride (Sigma) was dissolved in 40ml of sterile distilled water and the volume made up to 50ml with sterile distilled water. The solution was autoclaved and stored at room temperature.

70% Ethanol

70ml of pure Ethanol (BDH) was added to sterile distilled water and the volume made up to 100ml with sterile distilled water. The solution was stored at 4°C.

75% Ethanol

75ml of pure Ethanol (BDH) was added to sterile distilled water and the volume made up to 100ml with sterile distilled water. The solution was stored at 4°C.

TE buffer (10mM Tris-Cl, 1mM EDTA)

10ml of 1M Tris-Cl pH 7.5 and 2ml of 500mM EDTA pH 8.0 were added to sterile distilled water and the volume made up to 1000ml with sterile distilled water. The solution was autoclaved and stored at room temperature.

3M Sodium acetate (pH 5.2)

40.8g of Sodium acetate (Sigma) was added to 80ml sterile distilled water, pH was adjusted to 5.2 with glacial acetic acid and the volume made up to 100ml with sterile distilled water. The solution was autoclaved and stored at room temperature.

Phenol : Chloroform (1:1)

20ml of liquid phenol (Gen-Apex) was added to 20ml of Chloroform (R&M). The solution was stored in amber bottle at 4°C.

DNeasy Plant Mini Kit (Qiagen)

4.2.3 DNA Isolation Method

4.2.3.1 DNA Isolation Using Fresh Roots

0.5-1.0g of fresh roots that were rinsed with distilled water to remove foreign materials. The fresh roots were ground in liquid nitrogen using a mortar and pestle. The powder was transferred to a 50ml centrifuge tube containing 20ml of extraction buffer and centrifuged at 10,000g at 4°C for 10 minutes. Supernatant was decanted, upon centrifugation the pellet was dissolved in 20ml extraction buffer by inverting the tube. The mixture was then centrifuged at 10,000g at 4°C for 10 minutes. This step was repeated one more time.

Supernatant was decanted, the pellet was resuspended in 5ml extraction buffer, 3.5ml high-salt CTAB and 0.3ml Sarkosyl 30% were added to the tubes accordingly and incubated in water bath at 55°C for 60 minutes. Equal volume of chloroform : isoamyl alcohol was added to the tube and centrifuged at 10,000g for 10 minutes. Supernatant was transferred to a 50ml centrifuge tube, 2/3 volume of cold absolute isopropanol and 1/10 volume sodium acetate were added to the tubes accordingly and centrifuged at 10,000g at 4°C for 20 minutes. Supernatant was decanted, the pellet was washed with 75% cold ethanol. Supernatant was decanted, the pellet was air-dried and resuspended in 200 µl of TE buffer. 10µl of RNase stock solution was added to the tube and incubated in water bath at 37°C for 40 minutes. The solution was transferred to 1.5ml microcentrifuge tube, equal volume of phenol : chloroform was added and centrifuged at 14,000 rpm for 10 minutes at room temperature.

Upper aqueous phase was transferred to a new 1.5ml microcentrifuge tube; equal volume of cold chloroform was added and centrifuged at 14,000 rpm for 10 minutes at room

temperature. Upper aqueous phase was transferred to 1.5ml microcentrifuge tube, 2 volumes of absolute cold 100% ethanol and 1/10 volume of sodium acetate solution were added and kept the microcentrifuge tube at -20°C for 30 minutes. The pellet DNA was obtained by centrifuged at 14,000 rpm for 15 minutes, rinsed with cold 75% ethanol, air-dried and dissolved in 30-50µl of TE buffer.

4.2.3.2 Modified DNA Isolation Method Using Young Leaflet

In this method, 0.1 grams of young leaflet of germinated seeds were ground to fine powder in liquid nitrogen using a mortar and pestle. The powder was transferred to a microcentrifuge tube containing 700µl of DNA isolation buffer, gently inverted and incubated in water bath at 65°C for 45 minutes. The mixture was extracted twice using equal volumes of chloroform:isoamyl alcohol and the tubes were centrifuged at 10,000 rpm for 15 minutes. Supernatant was transferred to a new tube and 0.5 volumes of 5M NaCl and 2 volumes of absolute cold isopropanol were added, tube gently inverted and incubated on ice for 15 minutes. The tube was centrifuged at 10,000 rpm for 5 minutes and the resulting pellet was washed twice with 500µl of 70% ethanol. Pellet was air dried and dissolved in 30-50µl of TE buffer.

4.2.3.3 DNeasy Plant Mini (Qiagen) Using Young Leaflets

DNA was extracted from 0.1 grams of sample material. The sample was ground to fine powder in liquid nitrogen using a mortar and pestle. The powder was transferred to a microcentrifuge tube, 400µl of Buffer AP1 and 4 µl RNase A were added, vortexed and incubated in water bath at 65°C for 10 minutes. The tube was inverted 2-3 times during incubation. 130µl of Buffer AP2 was added to the mixture, mixed and incubated on ice for

5 minutes. The lysate was centrifuged at 14,000rpm for 5 minutes and the lysate was pipeted into a QIAshedder Mini spin column in a 2ml collection tube, centrifuged at 14,000rpm for 2 minutes. The flow-through fraction was transferred into a new tube without disrupting the pellet, 1.5 volume of Buffer AP3/E was added to the flow-through and mixed by pipetting. The 650µl of mixture was transferred into a DNeasy Mini spin column in a 2ml collection tube, centrifuged at 8,000rpm for 1 minute and flow-through was discarded. The spin column was placed into a new 2ml collection tube, 500µl Buffer AW was added to the spin column, centrifuged at 8,000rpm for 1 minute and flow-through was discarded. Another 500µl Buffer AW was added to the spin column and centrifuged at 14,000rpm for 2 minutes. The spin column was transferred to a 1.5ml microcentrifuge tube, 50µl Buffer AE was added to spin column, incubated for 5 minutes at room temperature and centrifuged at 8,000rpm for 1 minute. 50µl Buffer AE was added to spin column again, incubated for 5 minutes at room temperature and centrifuged at 8,000rpm for 1 minute.

4.2.4 DNA Quantification

1000X dilution was made by adding 2900µl of distilled water to the cuvette followed by 3µl of sample DNA (4.2.3.1, 4.2.3.2 and 4.2.3.3) and 97µl of distilled water. It was thoroughly mixed by pipetting. 3ml of distilled water was used as a blank. The absorbance was recorded at 260nm and 280nm. DNA concentration was calculated using the following equation: DNA concentration (µg/µl) = OD_{260} x dilution factor x (50µg DNA/ml). Purity was calculated by taking the ratio of reading of OD_{260} / OD_{280}

4.2.5 0.8% Agarose Gel Electrophoresis

0.4g of agarose (AppliChem) was added to 1ml of 50X TAE buffer and 49ml of sterile distilled water. The solution was boiled until the agarose is completely dissolved. The solution was removed after boiling and allowed to cool to 60°C. 1.5µl ethidium bromide was added and mixed by gently swirling the solution. The solution was poured into a casting tray containing a sample comb and allows it to solidify at room temperature. The tape and comb were removed after the gel has solidified. Aliquot of DNA (4.2.3.1, 4.2.3.2 and 4.2.3.3) was mixed with 6X loading dye (EURx) and loaded into the well to electrophoresis at 60V for 4 hours. The migration pattern was capture with a camera.

4.3 RESULTS

4.3.1 DNA From Using Fresh Roots

Isolation of genomic DNA using the described method, gave a DNA yield of 57.5µg/g of roots material. The pellet was brown in colour and the spectrophotometer readings which gave a A_{260} nm/A_{280} nm ratio of 1.28 (Table 4.1). The band from gel electrophoresis showed DNA degradation as shown in Figure 4.3.

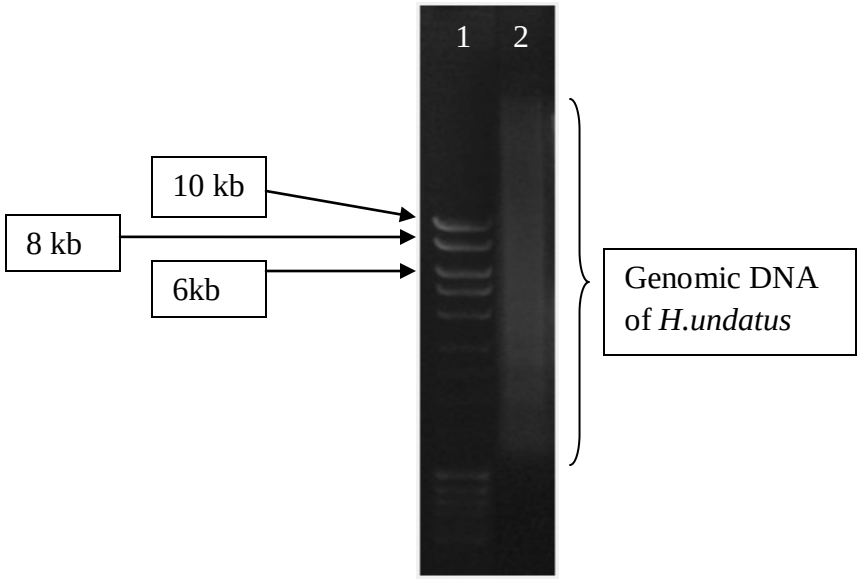

Fig 4.3: Electrophoresis of *H.undatus* genomic DNA from fresh roots on 0.8% agarose gel. Lane 1: Mass ruler, mix, DNA ladder and Lane 2 Genomic DNA of *H.undatus* from roots.

4.3.2 Modified DNA Isolation Method Using Young Leaflet

Isolation of genomic DNA using the described method, gave a DNA yield of 975 µg/g leaf material. The pellet was white in colour and the spectrophotometer readings which gave a A_{260} nm/A_{280} nm ratio of 1.92 (Table 4.1). The band from gel electrophoresis showed a sharp and intact band as shown in Figure 4.4.

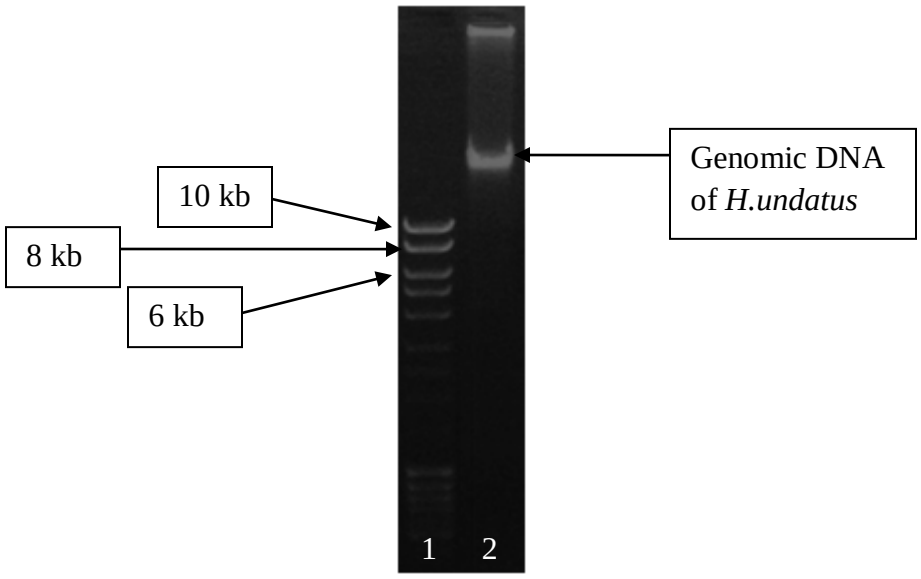

Fig 4.4: Electrophoresis of *H.undatus* genomic DNA from young leaflets on 0.8% agarose gel. Lane 1: Mass ruler, mix, DNA ladder and Lane 2 Genomic DNA of *H.undatus* from leaves using modified DNA isolation method.

4.3.3 DNeasy Plant Mini (Qiagen) Using Young Leaflets

Isolation of genomic DNA using the described method, gave a DNA yield of 200µg/g of leaf material. The pellet was white in colour and the spectrophotometer readings which gave a A_{260} nm/A_{280} nm ratio of 1.60 (Table 4.1). The band from gel electrophoresis showed a sharp and intact band as shown in Figure 4.5.

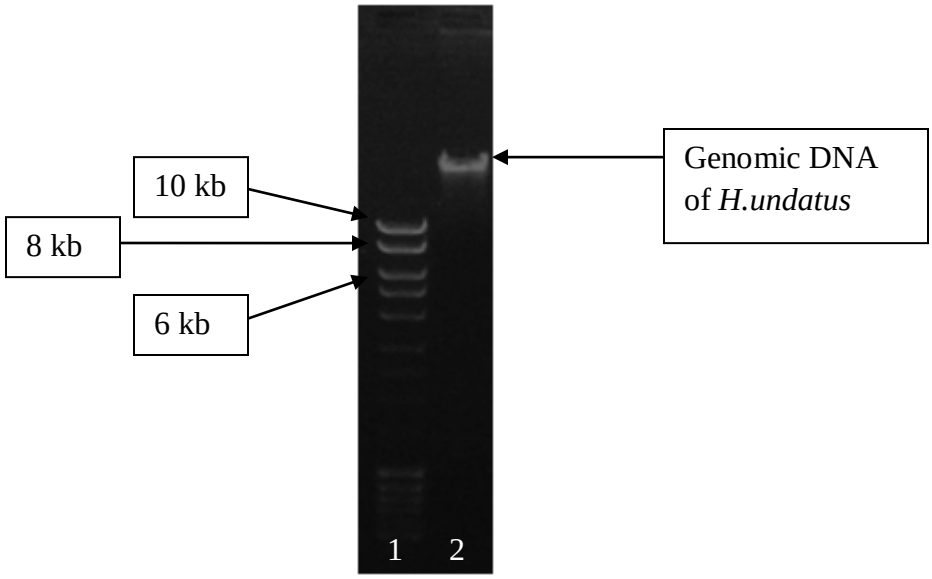

Fig 4.5: Electrophoresis of *H.undatus* genomic DNA from young leaflets using Qiagen kit on 0.8% agarose gel. Lane 1: Mass ruler, mix, DNA ladder and Lane 2 Genomic DNA of *H.undatus* from leaves using DNeasy Plant Mini (Qiagen).

Table 4.1: DNA analysis using UV-spectrophotometer. Table comparing purity using different starting materials.

	A_{260}	A_{280}	A_{260}/A_{280}
Young leaflets (Commercial kits)	0.008	0.005	1.60
Roots	0.023	0.018	1.28
Young leaflets	0.039	0.020	1.95

4.4 DISCUSSION

DNA isolation using young leaflets according to the modified DNA extraction protocol, gave the highest DNA yield of 975µg/g of leaf material as compared to the Tel-Zur *et al.,* 1999 DNA isolation method and DNeasy Plant Mini commercial kit (Qiagen) which had DNA yield of 57.5µg/g of roots material and 200µg/g of leaf material respectively. This yield is much better than compared to both methods and the purity of the DNA was within the desire range of 1.8-2.0. The result obtained using the Tel-Zur *et al.,* 1999 method using roots material could not be reproduced in this experiment. This may be due to different source of roots as it used roots cutting as opposed of using roots from the ground. Soil found on the roots may be another factor causing DNA degradation.

Both the extraction methods which used young leaflets as starting material able to produced intact DNA. However the DNA yield from the modified DNA extraction protocol was double of the yield from DNeasy Plant Mini commercial kit (Qiagen). This can be observed in Figure 4.4 and Figure 4.5; it was observed that some high molecular weight DNA was stuck in the well because of highly concentrated DNA.

H. undatus contains a high amount of polysaccharides which will bind to the DNA making it viscous and glutinous after the precipitation step during extraction. This will render the DNA unsuitable for downstream application such as PCR and restriction digest as the respective enzyme cannot access the DNA (Tel-Zur *et al.,* 1999, Barnwell *et al.,* 1998 and Puchooa, 2004). Presence of polysaccharide is easily detected as it imparts DNA extract to be sticky and viscous.

The DNA extraction method was adapted from Lodhi *et al.,* 1994 with a slight modification. The modification was the inclusion of Sodium Bis-sulfite, Sodium Sarkosyl and Sorbitol into the extraction buffer. Sodium Bis-sulphite prevents oxidation of phenolic compounds whereas Sodium Sarkosyl precipitates high levels of polysaccharides (Havania *et al.,* 2004 and Sharma *et al.,* 2002). Lodhi protocol was a tissues extraction with CTAB in high salt condition which suppresses the co-precipitation of polysaccharides and DNA. This protocol used 5ml of extraction buffer for 0.5g of leaves with a ratio of 1ml for every 1g of leave (Murray and Thompson, 1980 and Lodhi *et al.*, 1994). However, in this modified extraction protocol, only 0.7ml of isolation buffer was used for 0.1g which is sufficient to yield a high quality DNA. To obtain a better quality DNA, the isolation buffer was preheated to 65°C before being used to shorten the time from bringing the frozen tissue from -80°C to 65°C as suggested in Puchooa, 2004.

In the plant cells, the phenolic compounds are separated from DNA by compartmentalization. Once the cells are disrupted, the phenolic compounds will bind to DNA. The formation of oxidized phenolic compounds can be inhibited by the inclusion of low molecular weight of polyvinyl pyrrolidone (PVP) and antioxidants into the isolation buffer (Salzman *et al.,* 1999). PVP will form complexes with polyphenolics through hydrogen bonding and β-mercaptoethanol reduces oxidation of phenolic compounds (Micheils *et al.*, 2003, De la Cruz *et al.*, 1997 and Maliyakal, 1992 and Lodhi *et al.*, 1994). Examples of antioxidants include β-mercaptoethanol and ascorbic acid (Puchooa, 2004).

It took a week for the seeds to germinate and produce partially expanded leaflet. Young partially expanded leaves are the best material and were chosen as a starting material for

genomic DNA extraction (Lodhi *et al.,* 1994). This stage is crucial because fully expanded mature leave are heavily loaded with polysaccharide and polyphenols which will interfere with the DNA isolation. When the cells are disrupted, the contaminants (polysaccharide and polyphenols) will come into contact with DNA resulting in poor quality of DNA (Havania *et al.,* 2004). Besides that, mature leaves are tougher making the grinding process difficult (Couch and Fritz, 1990). The germination process is cheaper and easier as compared to generating callus.

In the grinding process, the leaves should be thoroughly ground but not into a very fine powder before adding the DNA isolation buffer. If the leaves were ground into a very fine powder, the DNA will be sheared. Furthermore, the pulverised tissue should not start to thaw before the inclusion of the DNA isolation buffer to prevent nucleolytic degradation of DNA. Slight thawing will cause a significant build up of polyphenolic complexes. Hence utmost care must be taken to insure that the frozen material never thaws prior to contact with the DNA isolation buffer (Couch and Fritz, 1990).

Seed germination provides a ready source of genomic DNA and using this described protocol, a high yield 975µg/g of leaf material DNA can be obtained. This result was reproducible and DNA can be stored in TE buffer at -20°C for months without degradation.