African Journal of Biotechnology Vol. 8 (13), pp. 3036-3040, 6 July, 2009
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2009 Academic Journals

Full Length Research Paper

Isolation of high quality DNA and RNA from cambium of the East African Greenheart (*Warburgia ugandensis*)

E. Muge[1]*, K. Burg[2], C. Kadu[1], A. Muchugi[1], S. Lemurt[1] and R. Jamnadass[1]

[1]World Agro forestry Centre (ICRAF), Box 30677-00100, Nairobi, Kenya.
[2]Austrian Research Centers GmbH - ARC Department of Bioresources, A-2444 Seibersdorf, Austria.

Accepted 14 May, 2009

The genus *Warburgia* belongs to the Canellaceae, a small family of tropical aromatic tree species important for their antibacterial and antifungal medicinal qualities amongst many other functions. Many published protocols for nucleic acid isolation failed to yield sufficiently good quality amounts for analysis. We have therefore developed a simple and fast CTAB (cetyl-trimethyl ammonium bromide)-based extraction protocol for routine isolation of high-quality nucleic acids. Good quality genomic DNA (gDNA) and RNA was obtained and was suitable for PCR amplification, restriction endonuclease digestion, Southern blotting and RT-PCR, respectively.

Key words: Nucleic acid extraction, polyphenols, polysaccharides, medicinal plants.

INTRODUCTION

Traditional medicine is a key element among the rural communities in developing countries and over 80% of these populations directly rely on phytomedicine for the provision of primary healthcare especially where there is an inadequate primary health care system (Tabuti et al., 2003; Shrestha and Dhillion, 2003; Ssegawa and Kasenene, 2007). Continued utilization of medicinal plant species is however threatened by among other things habitat modification and unsustainable rates of exploitation (Joshi and Joshi, 2000; Tabuti et al., 2003).

The genus *Warburgia* which *Warburgia ugandensis* belongs are extensively harvested due to their antibacterial and antifungal medicinal qualities, a fact attributed to their unique sesquiterpene dialdehydes (Olila et al., 2001; Beentje, 1994). The species are reported as becoming rare in areas where they were once plentiful (Beentje, 1994) and has been listed as vulnerable in the 'Red List' data in Southern Africa (Cunningham, 2001). With the current trend in the global use of herbal medicine and the reliance of local communities in sub-Saharan Africa on traditional medicine (Olila et al., 2001), continued exploitation of *Warburgia* species raises concerns regarding its long-term sustainability of utilization and conservation.

The World Agroforestry Centre (ICRAF) is promoting on-farm planting of medicinal trees as a means for conservation and sustainable use of tropical trees like Warburgia. Molecular biological approaches will contribute significantly in facilitating development, collection and conservation strategies of these medicinals.

High quality DNA and RNA is a prerequisite for molecular biological studies, however extracting nucleic acids from many tropical species can be problematic due to the variations in plant cell components, including polysaccharides, cellulose, and phenols (Woodhead et al., 1998; Porebski et al., 1997). Problems encountered in the isolation and purification of DNA and RNA from cambium could include those due to inhibitor compounds like polyphenols which are known to oxidize and covalently link with quinines and to bind nucleic acids (Loomis, 1974), other secondary metabolites as well as the difficulty of obtaining the tissue.

The tissue of choice was the cambia which is layer of actively dividing cells between xylem (wood) and phloem (bast) tissues responsible for the secondary growth of stems and roots. Obtaining this tissue from mature Warburgia stems and roots is quite difficult as it is very thin and protected by the hard dead outer bark. This study

*Corresponding author. E-mail: e.kirwa@cgiar.org. Tel.: +254-20-7224000. Fax: +254-20-7224001.

Abbreviations: CTAB, Cetyl-trimethylammoniumbromide; **RT-PCR,** reverse-transcription-polymerase chain reaction; **PVP**, Polyvinylpyrrolidone; **DEPC**, diethylpyrocarbonate.

was therefore aimed at optimizing a protocol that would within the limiting tissue availability, yield sufficient and good quality nucleic acids for downstream molecular studies involving PCR amplifications, restriction enzyme digestion among others in an attempt to better understand the composition and biosynthesis of medicinal compounds in *W. ugandensis* and other important medicinal trees. Here we describe an optimized rapid nucleic acid isolation protocol with a common extraction buffer that yields an enriched nucleic acid lysate for DNA and RNA isolation from fresh *Warburgia* cambium as the starting material.

MATERIALS AND METHODS

Plant material

Cambium from freshly harvested *Warburgia* tree barks was scrapped using sterile scalpel blade. The samples (≈ 0.5 g) each were put into 2 ml microfuge tubes and immediately frozen in liquid nitrogen.
All buffers except Tris-HCl, were prepared with Millipore-purified (MQ) water, treated with diethylpyrocarbonate (DEPC) (Sambrook et al., 2001) and autoclaved, unless indicated otherwise.

Isolation of tissue extract

Frozen cambium material (0.3 g) was ground into a fine powder using a mortar in presence of liquid nitrogen. The powder was transferred to a microfuge tube containing 700 µl extraction buffer (3% CTAB (w/v), 100 mM Tris-HCl (pH 7.5), 1.5M NaCl, 50 mM EDTA (pH 8.0), 0.2% β-mercaptoethanol (v/v), 1% polyvinylpyrrolidone (PVP-40) (w/v), 1% Sodium Sulphite (w/v)) pre-heated to 65°C after addition of 4 µl of Proteinase K (10 mg/ml). The contents were mixed by inversion then incubated at 37°C and 65°C (water bath) for 10 min each, respectively. Samples could be stored in this state for up to three days at room temperature before extraction. The lysate was extracted twice with 700 µl of chloroform/-isoamyl alcohol (24:1) and the aqueous phase separated by centrifuging at maximum speed (14,000 g) in a microcentrifuge for 15 min at room temperature. The aqueous phase after the second extraction was collected and used for either DNA or RNA isolation.

DNA isolation

DNA was directly precipitated from the aqueous phase by adding 1/10 volume 5M NaCl and an equal volume of ice-cold Isopropanol and incubated at -20°C for 1 h. The precipitated DNA was pelleted by centrifugation at 14,000 g, 5 min at room temperature and the pellet washed twice with 450 µl of 70% ethanol before air drying and re-suspending in 50 µl 1x TE [10 mM Tris-HCl (pH 8.0), 1 mM sodium-EDTA (pH 8.0)]. RNA contamination was removed by incubating the dissolved nucleic acids with 1/10 volume of RNase I (10 mg/ml) at 37°C for 30 min. The purified DNA quantity and quality was estimated spectrophotometrically by Nanodrop ND-1000 spectrophotometer (peQLab Biotechnologie GmbH, Erlangen- Germany) as well as standard molecular biology techniques (Sambrook et al., 2001). The preparations were stored either at 4°C or -20°C.

RNA isolation

The aqueous phase from the common step (tissue extract) was precipitated with ¼ volume of 10M LiCl overnight at 4°C. The precipitated RNA was pelleted by centrifugation (10,000 g, 4°C, and 20min.) then dissolved in 700 µl SSTE {1M NaCl, 0.5% SDS, 10 mM Tris-HCl (pH 8.0)} pre-warmed at 60°C and subsequently incubated at 60°C for 10 min. A two time extraction with Chloroform:isoamylalcohol (24:1) (v/v) was done followed by precipitation of the aqueous phase with 2xVol. absolute ethanol and incubated at -70°C for at least 1 h or at -20°C overnight. The precipitated RNA was pelleted at 13,000 g, 4°C for 30 min, washed twice with 70% ethanol, air dried and finally dissolved in 50 µl RNase free MQ water. The RNA quantity and quality was estimated spectrophotometrically by Nanodrop ND-1000 spectrophotometer (peQLab Biotechnologie GmbH, Erlangen- Germany) as well as standard molecular biology techniques (Sambrook et al., 2001).

Estimation of the quantity and quality of nucleic acids

The quantity and quality of nucleic acids was estimated spectrophotometrically by Nanodrop instrument (peQLab Biotechnologie GmbH, Erlangen Germany). The equipment gives the quantity in ng/µl hence the yield per gram of tissue can be calculated whereas the quality is determined by the absorbance ratios A260/230 and A260/280. The quality of both nucleic acids was further analysed by running standard agarose gel electrophoresis (1% and 0.8% gels for DNA and RNA respectfully). 15 µg of genomic DNA samples were digested with *EcoR*I, *Bam*HI, *Hin*dIII, *Ms*PI, *HP*aII and *Hae*III, restric-tion enzymes respectively at 37°C for 10 h and electrophoresed on a 0.8% in 1X TBE buffer containing 0.5 µg/ ml of ethidium bromide and visualized under UV light.

DNA PCR analysis

Polymerase chain reaction was performed using housekeeping genes primers Alpha tubulin Fwd 5'TTCAATGCTGTTGGTGGNG-G'3 and Rev. 5'TTGGCRTACAT-SAGRTCGAA'3, Beta-tubulin Fwd.5'AACAACTGGGCNAAGGGYCA and Rev.5'TCCTTGGTG-CTCATCTTTCC'3, Actin Fwd 5'AAACGGCTAC-CACATCCAAG and Rev. 5'AGACAAATCGCTCCACCAAC and Rev. 5'Cyclophilin Fwd 5'CAAAACSGC-GGAGAACTTCC'3 and finally, 18sRNA Fwd.5'ACTGGGATGAYATGGAGAAG and Rev. 5'AYCCTCCAAT-CCAGACACTG'3. These were designed from conserved consensus gene sequences from Potato, Pepper, Wheat and Maize. The amplification was performed in 25 µl reaction volume containing 50 ng DNA template, 400 µM of each dNTP, 400 nM each primer, 0.625 units KlearTaq polymerase (KBiosciences) and 1.2 x PCR buffer. The thermal cycler conditions were: 15 min at 95°C, 35 cycles of 45s at 94°C, 30 s at 55°C and 60 s at 72°C and finally 10 min at 72°C. The amplified products were electrophoresed on a 1% agarose gel in 1X TBE buffer containing 0.5 µg/ ml of ethidium bromide and visualized under UV light.

cDNA PCR analysis

cDNA was prepared using iScript™ cDNA Synthesis Kit (Bio-Rad) according to the manufacturers' instructions. The typical reaction mixtures contained 4 µl 5x iScript Reaction Mix, 1 µl iScript Reverse Transcriptase and 1 µg Total RNA template in a final volume of 20 µl. The thermal cycling conditions were: 5 min at 25°C, 30 min at 42°C, 5 min at 85°C and a final hold at 4°C. The resulting cDNA was used as a template in PCR reaction using 18S and Actin housekeeping genes primers. The PCR was carried out in a total volume of 25 µl consisting of 2 µl of template cDNA, 400 µM of each dNTP, 400 nM each primer, 0.625 units KlearTaq polymerase (KBiosciences) and 1.2 x PCR buffer. The thermal cycler conditions were: 15min at 95°C, 35 cycles of 45 s at 94°C, 10 s at 55°C and

60 s at 72°C and completing the reaction by 72°C for 10 min. For analysis, the cDNA and the PCR products were electrophoresed on a 1% agarose gel containing 0.5 µg/ ml of ethidium bromide and visualized under UV light.

Southern blot analysis

Southern blot analysis was carried out according to Sambrook et al. (1989) with few modifications. 15 µg of *W. ugandensis* genomic DNA were subjected to total digestion with *Eco*RI, *Bam*HI, *Hin*dIII, *Ms*PI and *HP*aII overnight. The digested samples were separated on a 0.8% agarose gel (TBE buffer). The gel's DNA was then blotted onto positively charged nylon membrane (Hybond N+) (Amersham Biosciences) following depurination, alkali denaturation and neutralization; hybridized overnight at 68°C using [32P] dCTP (Perkin Elmer) labeled PCR purified α-tubulin sequence of *W. ugandensis.* For probe preparation 742 bp *W. ugandensis* α-tubulin sequence (NCBI Accession no. FJ379594) was PCR amplified and eluted. After hybridization, the membrane was washed thoroughly using 2X SSC (3 M Sodium Chloride and 0.3 M Tri sodium citrate, dihydrate) and 0.1% Sodium dodecyl sulphate (SDS) at room temperature for 1 h and at 68ºC for another hour using 0.1X SSC, 0.1% SDS and then exposed to Kodak X-ray film, stored at -80°C for 3 days and subsequently developed.

RESULTS AND DISCUSSION

Our aim was to provide a tissue extract appropriate for both DNA and RNA extraction. Most protocols use different extraction buffers for each of the nucleic acids, the extraction buffer in the current protocol was made in such a way as to come up with a lysate enriched with both nucleic acids without compromising their stability.

It is important to harvest the cambia tissue fast and immediately freeze to prevent excessive degradation of the nucleic acids. The young and succulent stem and root however yielded much nucleic acid due to the ease of harvesting and abundance of the tissue as opposed to the old stem and roots.

The DNA isolation procedure is CTAB-based, modified from Doyle and Doyle, 1987. The extraction buffer contained PVP and β-mercaptoethanol to prevent oxidation of the secondary metabolites in the disrupted plant material. To remove the polyphenols quite abundant in *Warburgia,* PVP which forms complexes with polyphenolic compounds, was added and complex separated from nucleic acids by centrifugation. Although different percentages ranging from 1 - 4 of PVP has been proposed in several protocols (1% Khanuja et al., 1999; 2% Csaikl et al., 1998 and 4% Keb Llanes et al., 2002), the lowest concentration (1%) worked well in our case and hence higher concentrations were not attempted.

Sodium sulphite, not included in most protocols was used as a reducing agent for PCR inhibitors and to improve the yield and stability of nucleic acids (Baranwal et al., 2003). It also prevents the oxidation of nucleic acids upon the release of sap from cells and degradation during storage (Byrne et al., 2001). Unlike in the protocols by Chang et al., (1993) and Doyle and Doyle, (1987) where 2% and in Khanuja et al. (1999) where 2.5% CTAB detergent was used, a high percent (3%) was considered appropriate in our protocol when the initial extraction buffers with similar CTAB amounts gave inadequate results (results not shown). CTAB is used to separate polysaccharides known to interfere with several biological enzymes e.g. polymerases, ligases and restriction endonucleases. They form tight complexes with DNA creating a gelatinous pellet hence the embedded DNA is inaccessible to the enzymes (Sharma et al., 2002).

Sodium chloride facilitated their removal by increasing solubility in ethanol hence preventing co-precipitation with DNA (Schlink and Reski, 2002). Although higher concentration of sodium chloride have been used in other protocols (2.2 M, Aljanabi et al., 1999 and 4M, Tel-Zur et al., 1999), a concentration of 1.5 M NaCl and two time washes with ethanol was adequate in our case. Complete digestion with restriction endonuclease (Figure 2A) and PCR amplification with housekeeping genes primers Figure 3A indicate the absence of polysaccharide interference. The restriction digested DNA could be readily blotted and hybridised with ^{32}P labelled probe (Figure 2B). The average yield of clean intact DNA on the Nanodrop, was ≈15 µg per 100 mg cambium tissue (Figure 1A). The purified DNA was also sufficient for very sensitive techniques such as direct sequencing without further purification steps (data not shown).

As for *Warburgia* RNA, the yield from various protocols was also low, this included RNAeasy Plant Mini Kit (Qiagen) and Chang et al. (1993). The same extraction buffer used during DNA extraction was considered adequate. The conventional fractionation of the lysate in Chloroform:Isoamylalcohol and the preferential precipitation of RNA to DNA by using high ionic strength salt (10 M Lithium chloride) was then implemented. The integrity of *Warburgia* RNA was apparent upon ethidium bromide stained agarose gels (Figure1B). The average concentration of total RNA was about 8 µg from 100 mg of tissue. The quality of RNA was sufficient for RT-PCR analysis (Figure 3B).

The quantification of nucleic acids was done using a spectrophotometer (NanoDrop, Technologies Inc.). The Absorbance ratio $A_{260/230}$ was higher than 2.0 indicating pure products devoid of polyphenols. The $A_{260/280}$ ratio was in the range of 1.8-1.9 for DNA and 1.9-2.1 for RNA. This attests to the absence of protein contaminants.

The Nucleic acids isolation procedures presented here is an attempt to provide a simple solution to the difficulty experienced in extraction of nucleic acids from tropical trees (especially those known to possess highly valued medicinal properties). The difficulty is attributed to the abundance of phenolic compounds and a variety of secondary metabolites (Pirttilä et al., 2001). This difficulty also affirms the fact that optimization of nucleic acid extraction protocols for any new species of study is paramount (Jones et al., 1997).

The procedures described are simple, rapid, effective

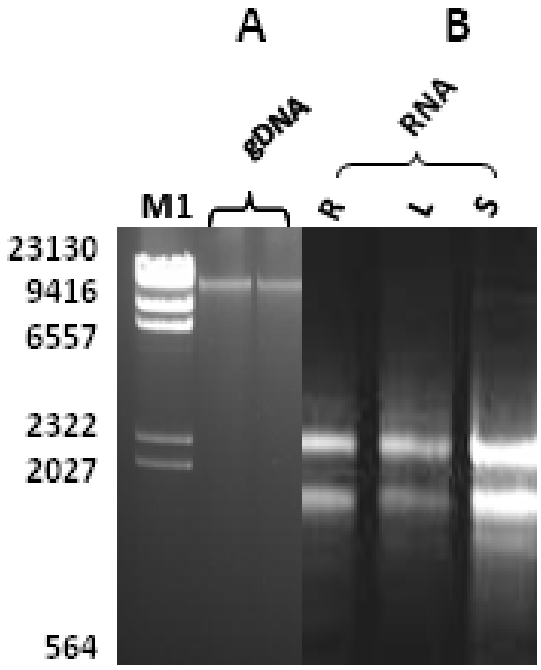

Figure 1. Electrophoretic analysis of cambia total genomic DNA (Figure 1a) and total RNA isolations (Figure 1b) from R (root) and S (stem) cambia as well as from L (leaf) of *Warburgia ugandensis.*

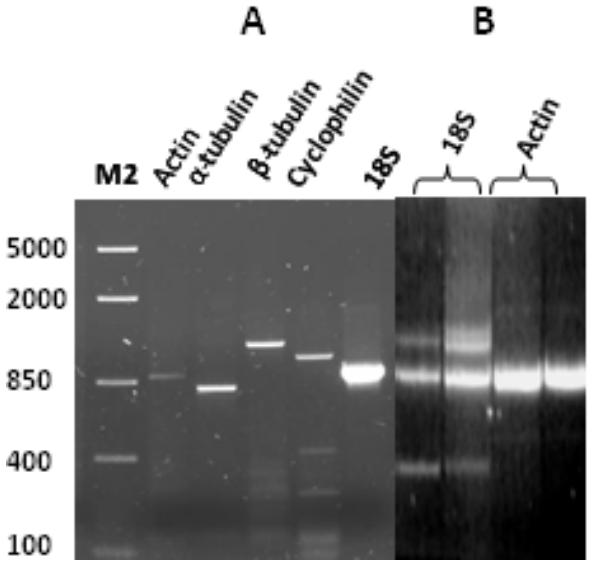

Figure 3. 1% agarose gel electrophoretic analysis of PCR products using total genomic DNA as a template with actin, α-tubulin, β-tubulin, cyclophilin and 18S degenerate primers respectively (Figure 3a) and with cDNA template with 18S and Actin degenerate primers (Figure 3b). M1 is FastRuler™ DNA Ladder, Middle Range (Fermentas).

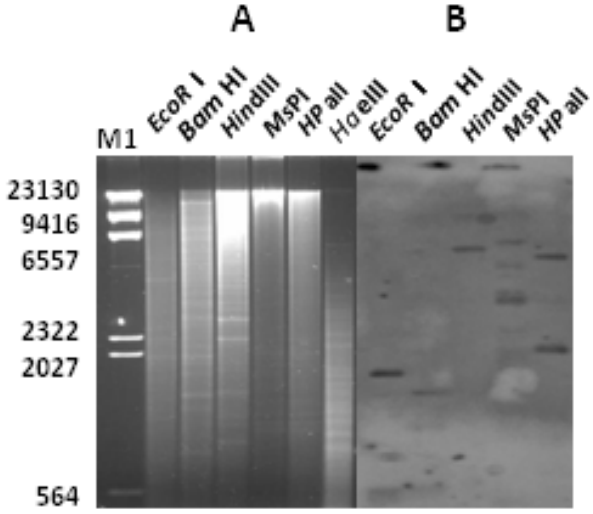

Figure 2. Restriction digestion (Figure 2a) and southern hybridization (Figure 2b) analysis of genomic DNA from *W. ugandensis*. M1 is Marker II DNA molecular weight marker (Roche) whereas the restriction enzymes used are shown above each lane. For clarity, the grey scale of the ethidium bromide-stained gel for southern hybridization was inverted.

and reproducible and can be scaled up as desired. Using this method, we subsequently had success in DNA extraction from leaves of *Warburgia* as well as from Allanblackia (*Allanblackia stuhlmannii*) that also posed problems for DNA extraction (results not shown). The stability of nucleic acids in the buffer could act as a putative storage means where time is limiting hence appropriate for short storage for transport and economical where cold storage facilities are limited. We consider as an advantage that principally, similar tissue extract can be used for DNA and RNA extraction from *Warburgia.* These protocols will hopefully provide an avenue towards genomic studies on *Warburgia* tree species as there is currently limited sequence data available despite the increasing interest towards its medicinal properties.

REFERENCES

Aljanabi SM, Forget l, Dookun A (1999). An improved and rapid protocol for the isolation of polysaccharide and polyphenol free sugarcane DNA. Plant Mol. Biol. Rep. 17: p. 281.

Baranwal VK, Majumder, S, Ahlawat, YS, Singh, RP (2003). Sodium sulphite yields improved DNA of higher stability for PCR detection of Citrus yellow mosaic virus from citrus leaves. J. Virol. Methods, 112: 153-156.

Beentje HJ (1994). Kenya trees, shrubs and lianas. Nairobi, Kenya; National Museums of Kenya, ISBN 9966986103, p. 722,

Byrne N, Macdonald B, Francki M (2001). Incorporation of sodium sulphite into extraction protocol minimizes degradation of *Acacia* DNA. Biotechniques. 30: 742-743.

Chang S, Puryear J, Cairney J (1993). A Simple and Efficient Method for Isolating RNA from Pine Trees. Plant Mol. Biol. Rep. 11: 113-116.

Csaikl U, Bastian M, Brettschneidder R, Gauch S, Meir A, Shauerte M, Scholz F, Sperisen C, Vornam B, Ziegenhagen B (1998). Comparative analysis of different DNA extraction protocols: A fast universal maxi preparation of high quality plant DNA for genetic eval-

uation and phylo-genetic studies. Plant Mol. Biol. Rep. 16: 69-86.
Cunningham T (2001). Return of the pepper bark. Medicinal Plant Conservation, 7: 21-22.
Doyle JJ, Doyle JL (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochemical Bulletin, 19: 11-15.
Jones CS, Iannetta PPM, Woodhead M, Davies HV, Mcnicol RJ, Taylor M A (1997). The isolation of RNA from raspberry (*Rubus idaeus*) fruit. Mol. Biotechnol. 8: 219-221.
Joshi AR, Joshi K (2000). Indigenous knowledge and uses of medicinal plants by local communities of the Gali Kandaki Watershed Area, Nepal. J. Ethnopharmacol. 73: 175-183.
keb llanes M, Gonzalez G, Chi-manzanero B, Infante D (2002). A rapid and simple method for small scale DNA extraction in Agavaceae and other tropical plants. Plant Mol. Biol. Rep. 20: 299a-299e.
Khanuja SPS, Shansany AK, Darokar MP, Kumar S (1999). Rapid isolation of DNA from dry and fresh samples of plants producing large amounts of secondary metabolites and essential oil. Plant Mol. Biol. Rep. 17: p. 74.
Loomis WD (1974). Overcoming problems of phenolics and quinones in the isolation of plant enzymes and organelles. Methods in Enzymol. 31: 528-545.
Olila D, Olwa-odyek, B, Opuda-asibo J (2001). Antibacterial and antifungal activities of extracts of *Zanthoxylum chalybeum* and *Warburgia ugandensis*, Ugandan medicinal plants. Afr. Health Sci. 1(2): 66-72.
Pirttilä AM, Hirsikorpi M, Kämäräinen T, Jaakola I, Hohtola A (2001). DNA isolation methods for medicinal and aromatic plants. Plant Mol. Biol. Rep. 19: 273a-273f.
Porebski S, Bailey IG, Baum BR (1997). Modification of a CTAB DNA extraction protocol for plants containing high polysaccharide and polyphenol components. Plant Mol. Biol. Rep. 15: 8-15.
Sambrook J, Fritsch EF, Maniatis T (1989). Molecular Cloning: A laboratory manual Second Edition Cold Spring Harbor Laboratory Press, Cold spring harbor, NY, USA pp. 9.31 - 9.58.
Sambrook J, Maccallum P, Russel D (2001). Molecular cloning: A laboratory manual, 3nd ed. Cold Springs Harbour Press, NY, ISBN 0-87969-577-3, p. 2344.
Schlink K, Reski R (2002). Preparing high-quality DNA from Moss (*Physcomitrella patens*). Plant Mol. Biol. Rep. 20: 423a-423f.
Sharma AD, Gill PK, Singh P (2002). DNA isolation from dry and fresh samples of polysaccharide-rich plants. Plant Mol. Biol. Rep. 20: 415a-415f.
Shrestha PM, Dhillion SS (2003). Medicinal plant diversity and use in the highlands of Dolakha District, Nepal. J. Ethnopharmacol. 86: 81-96.
Ssegawa P, John M, Kasenene JM (2007). Plants for malaria treatment in Southern Uganda: Traditional use, preference and ecological viability, J. Ethnobiol. 27: 110-131.
Tabuti JRS, Dhillion SS, Lye KA (2003). Traditional medicine in Bulamogi county, Uganda: its practitioners, users and viability. J. Ethnopharmacol. 85: 119-129.
Tel-zur N, Abbo S, Mylaboski D, Mizrahi Y (1999). Modified CTAB procedure for DNA isolation from epiphytic cavcti of genus *Hylocereus* and *Selenicerus* (Cactaceae). Plant Mol. Biol. Rep. 17: 249-254.
Woodhead M, Davies HV, Brennan RM, Taylor MA (1998). The isolation of genomic DNA from black curran (*Ribes nigrum* L.). Mol. Biotechnol. 9: 243-246.