SIMPLE DNA ISOLATION PROTOCOL FOR THE TUBEROUS CROP, *Gloriosa superba* L.

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Abstract. The isolation of intact and high-molecular mass genomic DNA is an initial and essential step for many molecular biology applications like restriction enzyme fingerprinting, polymerase chain reaction (PCR) etc. The presence of essential oils, polysaccharides, polyphenols and other secondary metabolites in the medicinal plants can hamper and reduce the yield and purity of extracted DNA. In this protocol, we describe simple modifications to the conventional cetyl trimethylammonium bromide (CTAB) method for reliable isolation of high molecular weight genomic DNA from *Gloriosa superba* - a potent medicinal plant. The purity of genomic DNA was confirmed by excellent absorbance (A260/A280) ratio of ~1.8 calculated from the spectrophotometric readings and the DNA concentration ranged from 75 to 308 µg/g of leaves. The PCR amplification from the isolated DNA was optimized to produce clear amplification in all the germplasm tested as mentioned in our earlier research. Besides being rapid, effective and technically easy, the protocol can also be used for the isolation of genomic DNA from other crop plants too.

Key words: *Gloriosa superba*; extraction; genomic DNA; PCR.

INTRODUCTION

Medicinal plants produce a wide diversity of phytochemical constituents which exhibit a wide array of pharmacological properties to treat infections, diseases or health disorders, or as spices, perfumes, toxins and pesticides [12, 39]. Based on their functions in fundamental metabolic processes, the phytochemical constituents are divided into two groups, namely primary and secondary metabolites. Primary plant metabolites are more or less identical in all living cells and are involved in metabolism, growth, and development in basic life processes. It consists of tiny molecules such nucleic acids, proteins, amino acids, polysaccharides, and tricarboxylic acids. Secondary metabolites are biologically active natural compounds with a wide range of structural compositions and often low molecular weight and are classified according to their chemical structures into several classes *viz* phenolics, alkaloids, saponins, terpenes [15, 27, 33, 40].

Gloriosa superba L. (family Colchicaceae) also known as as the flame lily or glory lily, is perennial climbing herb with tuberous roots from tropical Africa, India and south-eastern Asia. It is a poisonous herb containing high concentrations of colchicine in all its parts. It is commercially grown for use in Siddha, Ayurveda and Unani system of medicine and as a cash crop for extracting contains wide varieties of phytochemicals mainly 1,2-didemethyl colchicine, 2,3-didemethyl colchicine, colchicoside, salicylic acid, glucosides and flucoside being the most common [16, 20]. It is used for the treatment of gout arthritis, skin diseases, familia mediterranean fever, respiratory, urinary and reproductive systems, cardiovascular issues and many other disorders. It is both the state flower of Tamil Nadu (India) and the national flower of Zimbabwe [41].

The genetic improvement processes include screening of accessions, choosing of parents and selection of progeny. The isolation of intact, high-molecular mass genomic DNA is essential for crops improvement strategies including rDNA technology, endonuclease digestion, genomic library construction, southern blot analysis and polymerase chain reaction (PCR) technique like random amplified polymorphic DNA (RAPD), simple sequence repeats (SSR), inter simple sequence repeat (ISSR), Restriction Fragment Length Polymorphism (RFLP), Amplified fragment length polymorphism (AFLP), Short tandem repeats (STR) and in molecular biology applications. Furthermore, the germplasm characterization of medicinal tuberous crops facilitates the breeding of improved genotypes by utilizing molecular markers to identify the genetic links between accessions of the wild and cultivated gene pools [3, 35]. Selecting an appropriate method for DNA isolation requires a number of variables, including tissue type, DNA concentration, the purity needed for downstream applications, time, and cost [23]. The most basic steps involved in DNA purification from cellular components involves disruption, lysis, removal of proteins/ contaminants and recovery of DNA [31]. So, the efficiency of genomic DNA extraction from various plant species needs to be optimized to each plant tissue. It is challenging to extract high-quality DNA because these secondary metabolites which interfere by precipitating with the DNA, lowering the yield and lowering its quality [1, 14]. Removal of these substancrs requires isolation, quantitation as well as purification of the DNA using various organic solvents and alcohols.

Therefore, the present investigation was undertaken to obtain sufficient and high-quality genomic DNA from the tuberous medicinal plant *G. superba* for use in future research.

MATERIALS AND METHODS

Plant material. Plant material. Three grams leaves were collected from actively growing parts of *G. superba* plantlets maintained in the green house at Department of Botany, Kurukshetra University, Haryana (India) and immediately brought to the laboratory.

DNA isolation procedure. The leaves were washed with distilled sterile water and cleaned with moist tissue paper. The method described here was developed by introducing the following modifications in CTAB method [7, 29, 30]. The leaf sample (2-3 g) was grounded in liquid nitrogen (–196 °C) using a sterile mortar-pestle. The homogenized material was put into 15 mL of pre-warmed (60°C) DNA isolation buffer (2× CTAB extraction buffer comprising 100 mM Tris, 1.4 M NaCl, 20 mM EDTA, 2% (w/v) CTAB and 2 µL/mL β-mercaptoethanol) in capped polypropylene tubes. After that, a spatula was used to suspend the clump. It was then incubated for 1 hour at 60°C with occasional mixing by gentle swirling in water bath. After removing from water bath, an equal volume (15 mL) of Chloroform:isoamyl alcohol (24:1, v/v) was added and mixed gently for 15 minute by slight inversion to ensure phase emulsion. It was then followed by centrifugation at 15000 rpm for 10 minutes to recover aqueous phase in another tube. The extraction steps were repeated until the upper aqueous phase became clear. A two-thirds volume of ice-cold iso-propanol was added to a aqueous phase, and incubated at -20°C for 30 min and then centrifuged at 15000 rpm for 15 minutes at 20°C. The supernatant was discarded without disturbing the pellet. A 20 mL of 70% ethanol was added to DNA pellet and was gently stirred for 20 minutes. The pellet was collected by centrifugation (at 20°C) for 5 minutes at 10000 rpm. The tubes were inverted on paper towel to drain. The pellet was covered with parafilm with tiny pores and allowed to dry over night. The pellet was re-dissolved in 100 µL of $T_{10}E_1$ buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8) by keeping overnight at room temperature without agitation.

Purification of DNA: RNA contamination can be removed by treating the sample with DNase-free RNase. Protein including RNase can be removed by using a mixture of chloroform: Isoamylalcohol (24:1). Add 2.5 µL of RNase to 0.5 mL of crude DNA preparation (2.5 µL RNase = 25 µg RNase, so the treatment was 50 µg/mL of DNA preparation) and mixed gently. The content was gently mixed thoroughly and incubated at 37ºC for 1 h. A mixture of 0.3-0.4 mL of chloroform: isoamyl alcohol (24:1) was added, mixed well and centrifuged for 15 minutes at 15000 rpm. The supernatant was taken out avoiding the whitish layer at interface. The DNA was re-precipitated by adding double quantity of absolute alcohol. The tube was centrifuged at 5000-10000 rpm for 10 minutes to pellet the DNA. The transparent and viscous pellet was washed with 70% alcohol and allowed to dry overnight. The DNA was redissolved in 200 µL of $T_{10}E_1$ buffer.

Gel analysis: DNA purity was tested by loading extracted genomic DNA samples onto a 150 mL casted agarose gel (0.8%) in 0.5 x TBE (Tris borate EDTA) buffer containing (0.5 µg/mL) of ethidium bromide. A 5 µL of DNA sample was loaded with a mixture of DNA loading dye. A known amount of uncut λ phage DNA as control was loaded in the adjacent well. The gel was electrophoresed at 50 V for 1.5 hours. The gels were visualized and photographed under UV light.

Quantitation of DNA: The quantification of genomic DNA was achieved by observing it at 260 nm and 280 nm wavelengths using a spectrophotometer (NanoDrop ND-1000 version V3.1.1). A 2 µL of double distilled water sample was loaded in the lower pedestal to initialize the spectrophotometer. Fixed wavelengths of 260 and 280 nm were used. A 2 µL of $T_{10}E_1$ was loaded for the blank measurement.

Dilution of DNA for PCR: The quantified genomic DNA obtained was diluted to 25 ng/µL in $T_{10}E_1$, buffer (1mM EDTA,10 mM Tris-HCl, pH 8.0) and was subjected to PCR amplification. The samples were stored for further study at 4ºC. To check the suitability of extracted DNA for downstream analysis, 50 RAPD and 30 ISSR primers were screened [41].

RESULTS

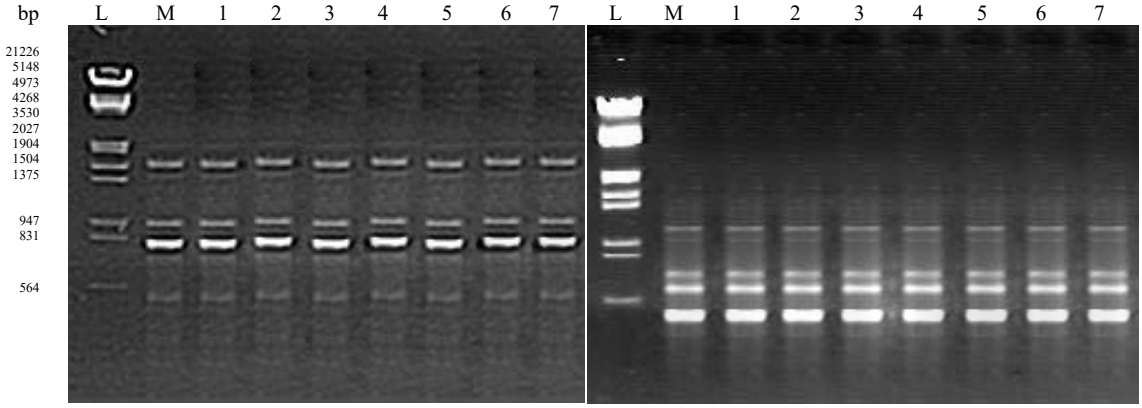
Quantification of DNA is commonly used to determine the average concentrations of nucleic acid present in the mixture. In this research, we have successfully isolated good yield of DNA from young leaves of *G. superba* (mother plant and micropropagated plants). Quantity and quality of the DNA samples were evaluated by measuring absorbance at 260 nm and 280 nm using UV-spectrophotometer. The A260/A280 ratio can be a useful tool to indicate the purity of DNA. A ratio of ~1.8 is generally accepted as pure DNA, devoid of impurities like proteins, polysaccharides and RNA. The amount of DNA isolated from various samples ranged from 75 to 308 µg/g of leaves (Table 1).

The quality of DNA was also examined using gel electrophoresis (0.8 %) revealing a single discrete band at the corresponding position to λ phase DNA, demonstrating that isolated genomic DNA was of high quality and had high molecular weight (greater than 10000 base pairs). It was intact, also free from mechanical or enzymatic degradation and RNA contamination.

The suitability of the extracted DNA was checked for downstream analysis using PCR based markers, of which only 10 RAPD and 7 ISSR primers yielded a total of 98 clear, distinct and reproducible amplicons, as described in our previous study [41] (Fig. 1). Finally, our protocol is simple, rapid, inexpensive and yields high DNA concentration using low amount of plant tissue.

Table 1. Quantity and quality of isolated genomic DNA of *G. superba* leaves.

Samples	A_{260} nm	A_{280} nm	Ratio of A_{260}/A_{280}	Conc. of DNA (µg/µL)	Yield in µg/g of leaves
Mother Plant	0.30	0.20	1.5	0.75	125
Plant 1	0.28	0.14	2.0	0.70	116
Plant 2	0.74	0.40	1.8	1.85	308
Plant 3	0.37	0.22	1.7	0.92	154
Plant 4	0.22	0.12	1.8	0.55	92
Plant 5	0.28	0.16	1.7	0.70	116
Plant 6	0.56	0.29	1.9	1.40	233
Plant 7	0.18	0.11	1.7	0.45	75



A. RAPD-GCC139 **B.** ISSR-GCC847

Figure 1. PCR profiles of *G. superba* plants with primers **(A)** RAPD- GCC139; **(B)** ISSR- GCC847
Lane L: represents the 1-kb ladder; lane M, 1-7: *G. superba* plantlets.

DISCUSSION

DNA isolation is a fundamental requirement in the field of plant molecular biology [19]. The quality of the isolated DNA directly influence the findings of each and every subsequent scientific research [24, 31]. Earlier, the process of DNA isolation and purification were used to be difficult and time consuming but currently many specialised methods based on different secondary metabolites and suitable buffer have been developed [28].

A suitable choice of leaf tissue is important to obtain high-quality DNA. Meristmatic region having the most dividing and young cells is the right choice due to their low concentration of secondary metabolites [5, 26]. UV spectrophotometers are commonly used to estimate the purity of a solution of nucleic acids [25]. The concentration of NaCl in buffer helps in the removal of proteins that are attached to the DNA, while EDTA is a chelating agent commonly used to eliminate contaminating divalent cations and to inhibit protease activity with less DNA degradation [9]. Strong reducing agents like β-Mercaptoethanol that acts on disulfide bonds are necessary for denaturing proteins while causing less DNA deterioration [34]. The extraction buffer causes lysis of membranes and the release of DNA from cellular organelles [37, 38]. The separation of contaminants into the organic phase and genomic DNA into the aqueous phase is facilitated by a mixture of chloroform and isoamyl alcohol [8, 23, 29]. The optimization of plant tissue quantity per unit volume of extraction buffer has an impact on genomic DNA isolation. A proper balance between the amount of plant tissue and the volume of the extraction buffer would reduce the the possibility of interfering of contaminants with the DNA pellet [21, 23].

The most efficient method for resolving DNA fragments according to their molecular weight or conformation in a solid support medium is agarose gel electrophoresis. The intact band of genomic DNA was observed in the gel by staining with ethidium bromide [22]. DNA samples are loaded into wells, seen as dark slots at the one end (top) of the gel. During this process, smaller fragments migrate faster than larger ones because the gel has sieving properties so that DNA molecules of different sizes are separated into distinct bands [32].

The success of the DNA purification depends on the restriction and ease-of-use of enzymes like polymerase and ligase. However, the presence of different polyphenolic compounds, polysaccharides, alkaloids, tannins and other metabolites in medicinal plants produce a gelatinous mass with nucleic acids, thereby inhibiting the action of *Taq* polymerase [15]. Similarly, mucilage, a polar glycoprotein found in tubers and seeds, also interferes the accurate pipetting of DNA, which causes volumetric errors [10]. The efficacy of isolated genomic DNA, devoid of interfering compounds, was further validated by PCR amplification or restriction digestion. PCR is a reliable method for *in vitro* selective amplification of certain genomic segments [13]. A standardized DNA isolation approach became necessitate in order to examine the genetic integrity of *in vitro* plants [4, 6, 11, 17, 18]. Our findings confirmed that the DNA was devoid of contamination with polysaccharides and polyphenols,

which might otherwise inhibit Taq DNA polymerase and restriction endonucleases from working properly [2, 14]. Such downstream molecular manipulations are not possible without high quality DNA [36].

In conclusion, the prescribed modifications in the this experimental study has established a basic, rapid, and effective standardized procedure for DNA extraction from medicinal crops which are rich in secondary metabolites. These modifications can be of great value for producing pure and high-quality DNA appropriate to be used for further molecular investigations. Moreover, it highlights its applicability for various other applications for management of genetic resources, biodiversity conservation, cultivar identification, genetic variability, phylo-genetic relationships and in genome mapping.

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