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ISOLATION AND PCR AMPLIFICATION OF GENOMIC DNA FROM LEAF SAMPLE OF *GLORIOSA SUPERBA* – A CONSERVATION APPROACH

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ABSTRACT:

Efficient method for isolation of high quality genomic DNA is the first step in the development of DNA based markers for genetic diversity study. Present study described a simple and efficient method for isolation of genomic DNA from leaves of *G. superba*. The modified method yields good quality, high molecular weight DNA, which is free from contaminants and coloured pigments. It is consistently amenable to PCR amplification and restriction digestion. This makes its wide use in various molecular biology applications *i.e.* genetic diversity study of the plant - as a conservation approach.

KEY WORDS: *G. superba, Genomic DNA, PCR, Amplification, Genetic diversity, Conservation.*

INTRODUCTION:

Genetic diversity had been studied in many plants using molecular DNA markers for resolving phylogenetical relationship, variability among the species, hybrid population and tissue cultured raised plants to identify elite plant. Genomic DNA is the base material for all these studies. Various protocols have been developed for isolating high quality of DNA from different plant species. However, the fundamental of DNA isolation always remains same. Most of the protocols developed, are based on chemical composition of the plant tissues used. It is known that each plant has specific

chemical make-up and hence requires specific treatment.

Isolating pure and amenable high-molecular-weight genomic DNA from plants is a crucial step as they contain high levels of tannins, polysaccharides, polyphenols, and other secondary metabolites (F. R. H. Katterman and V. L. Shattuck,1993; D. G. Peterson *et al.,* 1997) and highly acidic nature of tissue extracts (D. Warude *et al*., 2003). Presence of polyphenolics hampers DNA isolation, restriction, amplification and cloning reactions. Several protocols for isolation of genomic DNA contain high amounts of polyphenolics and polysaccharides (K. Burr, R *et al*., 2001; C. S. Kim *et al*., 1997). This problem is overcome by using modified CTAB protocol (C. S. Kim *et al*., 1997; S. Porebski *et al.,* 1997). However, it impart brown colour and reduce the yield and purity of isolated DNA (F. R. H. Katterman and V. L. Shattuck, 1993; S. M. Aljanabi *et al.,* 1999; P. Guillemaut and L. Maréchal-Drouard, 1992). Sometimes, due to chemotypic heterogeneity among plant species, a single DNA extraction protocol for all plant could not allow optimal yield of DNA [P. Sharma, *et al.,* 2010]. Not only for plant, but it also differs with plant tissue used. Thus, even closely related species requires different isolation protocol (K. Weishing *et al*.,1995).

Established protocols are variants of a few principal protocols (S. L. Dellaporta *et al*., 1983; J. J. Doyle and J. L. Doyle, 1987). However, they are sometimes not suitable for isolation of high quality DNA and yields low quality DNA which is not useful for further work. Hence, DNA isolation protocol should be optimized to each species and even to each tissue used (N. S. Sangwan *et al*.,1998)

Now a days, automatic nucleic acid extractors are available, which use silica column, or magnetic beads to isolate genomic DNA. FTA^{TM} paper based technique is also an alternative for this purpose (M. N. Mbogori *et al*., 2006), which overcome problems raise by manual isolation of DNA. However, it affects overall cost of the work; manual DNA isolation method with some modifications was preferred in present work.

Gloriosa superba, a perennial tuberous climbing herb belongs to family Liliaceae, is commonly known as *Karihari* / *Languli*. It is widely believed to have magical medicinal properties and is due to the presence of alkaloids in all parts of the plant. As it is unique in its kind, present study was aimed to develop a successful DNA isolation protocol, which is suitable for RFLP, RAPD analysis, restriction digestion and cloning experiments. This will be used as a conservation approach of the plant.

MATERIAL AND METHODS:

Plant material:

Fresh leaves were collected from its natural growing habitat of Mehsana district of Gujarat, India. Collected plant materials were brought to the laboratory under cold temperature to prevent degradation and stored at -20°C until further analysis. 1 g leaves were used for isolation of genomic DNA.

DNA isolation:

Following pre-treatments were applied to isolate total genomic DNA.

a) 1 g leaves were ground in liquid nitrogen to make a fine powder using pre-chilled mortar and pestle. The fine powder was used for DNA isolation (D. Dhakshanamoorthy and S. Radhakrishanan, 2009).

b) 1 g leaves were dipped in 5 ml of fixing solution for 30 min to denature enzymes at room temperature (P. Sharma,*et al*.,2010). Treated tissue was removed from fixing solution and homogenized with pre-chilled mortar and pestle. It was used for further process.

Two DNA isolation methods, Doyle *et al.* (1987) and Dellaporta *et al*. (1983), with some modifications were applied. Different concentration of polyvinyl pyrrolidone (PVP), 1.5, 2.0 and 2.5% (P. Sharma,*et al*.,2010) and an additional step with chloroform and iso-amyl alcohol (24:1) in CTAB method (J. J. Doyle and J. L. Doyle,1990) for efficient removal of polyphenol and polysaccharides were applied (Table I). The precipitation step was repeated. DNA was suspended in 50µl of Tris-EDTA (TE) buffer (pH 8.0). Extracted DNA was checked for its quality, quantity and amenable PCR amplification characteristic and hence, following parameters were applied.

1. DNA visual observation:

Extracted DNA from above mentioned methods was classified according to visual inspection of coloration (colourless, yellowish or dark).

2. DNA quantification:

To estimate the quantity and quality (in terms of protein and RNA contamination) of isolated genomic DNA, biophotometer was used. Absorbance at λ_{260} and λ_{280} and absorbance ratio (λ_{260}/ λ_{280}) was noted down, which indicates purity of DNA (J. Sambrook,*et al*.,1989).

3. DNA quality verification:

To check the form of DNA (linear or sheared) and RNA contamination of isolated genomic DNA, electrophoresis at 100 V was done using (0.8%) agarose gel and stained with 10 mg/ml ethidium bromide in 1X TBE buffer. Gel was visualized under UV light (of gel documentation system) and photographed. Quality was judged by viewing the image of single compact DNA band. Isolated DNA was used as template DNA for PCR to check either it is amenable or not.

PCR Amplification:

DNA amplification was performed as mentioned in (J. G. K. Williams, *et al*., 1990) with some modifications using specific primers. Optimum PCR conditions for amplification of DNA were assembled (data not shown). The amplified (PCR) products were subjected to agarose gel (1.5% [w/v]), prepared in 1X TBE with 3µl ethidium bromide (1mg/ml) staining. The 100bp standard DNA molecular weight marker was run along with the samples to compare the molecular weight of amplified products (not shown). Electrophoresis was carried out at 120 V constant current to separate the amplified DNA

products/bands. The separated bands were visualized under gel document system and were photographed. PCR reaction was conducted thrice to ensure the reproducibility of the results.

Restriction digestion:

Genomic DNA was restricted by *Eco*R I (3U of enzyme/ µg of DNA, incubated overnight at 37 ^{0}C). After incubation, DNA was run on (0.8%) agarose gel.

RESULTS AND DISCUSSION:

In any molecular biology work, the quality of DNA is more important than quantity of it. Isolation of quality DNA from *G.superba* is very difficult because of the presence of higher amount of polyphenolics and other secondary metabolites. In the study, two pre-treatments i.*e.* liquid nitrogen and absolute alcohol, and two different isolation methods with some modifications were applied. The basic difference between the methods used is that one was used phenol (S. L. Dellaporta *et al*., 1983) while the other one, it was excluded (J. J. Doyle and J. L. Doyle, 1987).

Higher molecular weight genomic DNA resulted after treating leaves in liquid nitrogen and absolute alcohol and isolating with CTAB protocol, described in (J. J. Doyle and J. L. Doyle, 1987) with some modifications. For isolation of purified DNA, different concentrations of PVP were employed, but it yielded best at 2.50%. Both pre-treatments were yielded good quality DNA (Fig. 1). Extracted DNA from above mentioned methods was classified according to visual inspection of coloration which indicated no oxidation of samples (colorless). The λ260/ λ280 ratio was near about 1.8 indicating high level of purity of isolated DNA. DNA yield ranged from 280-732 µg/g tissue. The DNA isolated following method described in (S. L. Dellaporta *et al*., 1983) showed the ratio (λ260/ λ280) was ranged from 1.09 to 1.65 indicating a wide range of quality (data not shown). This could be due to the presence of other impurities like proteins. Proteins sometimes associated with the isolated DNA and could not be removed by the treatment applied and may cause retardation in migration on agaroge gel. This is also supported in (S. Haque, A. *et al*., 2004).

The restricted DNA produced smear on (0.8%) agarose gel, indicates complete digestion of isolated DNA sample (Fig. 2).

The quality and quantity of isolated DNA was also reflected on agarose gel (Fig. 1). Isolated DNA was subjected to amplification with random primers. Amplified products were obtained. A uniform pattern of bands was obtained, which means that good quality of DNA was obtained.

DISCUSSION:

Two pre-treatment used in the present study showed differences in quality and quantity of genomic DNA isolated from *G. superba* leaves. In addition to fixing of the leaves in absolute alcohol, they were also ground in liquid nitrogen for comparison. The λ260/ λ280 with liquid nitrogen was in range 1.84-1.91

and with absolute alcohol, it was 1.42-1.78 (Table I). Thus, results do not indicate much difference in the quantity and quality of the genomic DNA isolated from leaves either fixed in alcohol or ground in presence of liquid nitrogen. However, the comparable protocol as observed may lead in saving of liquid nitrogen when alcohol fixed tissues are used for DNA extraction, especially in those laboratories where availability of liquid nitrogen is a limiting factor. In turn, the procedure becomes economical too.

The plant contains high amount of polphenolics, hence, higher concentrations (2.5%) of PVP was found suitable for successful isolation of DNA. It was also concluded in (D. Warude, *et al*., 2003), that high concentration of PVP was helpful to remove tannins and other polyphenolics from plant tissues. PVP and an additional step with chloroform and iso-amyl alcohol (24:1) in CTAB method recommended for efficient removal of polyphenol and polyssacharides by (D. Warude *et al*.,2003).

DNA isolation protocol described in (J. J. Doyle *et al.*, 1987) is more suitable than (S. L. Dellaporta, *et al*., 1983) with said modifications. It was noted in (D. Warude *et al*., 2003) that Dellaporta method (without phenol) is suitable for isolating DNA from seedlings and leaves but not from seeds. On the other hand Doyle *et al.*, (1987) method (with phenol) is effective for all tissues and also suitable for dead seed, when the sample is limiting (S. Haque, *et al*., 2004). Hence, the study also concluded that the method described by Doyle with some modification described, is very much suitable for the plant as less availability of material is sufficient for isolating good quality of DNA.

In *Chlorophytum* and *Azadirachta* maximum amount of DNA (1066 and 1820 µg/g tissue respectively) was obtained at room temperature when leaves were fixed in alcohol. Incubation at -80^{0}C impaired DNA quality compared to room temperature (P. Sharma *et al*., 2010).

The present procedure yielded high molecular weight DNA after grinding leaves in liquid nitrogen as well as absolute alcohol fixed leaves in CTAB. Isolated genomic DNA was amplified using specific primers and good amplification was observed (Fig. III). The results are in accordance to (P. Sharma *et al*., 2010, D. Dhakshanamoorthy and S. Radhakrishanan, 2009), that good quality of DNA can be isolated without the use of liquid nitrogen from different plant species.

The genomic DNA extracted from *G superba* was subjected to specific primer amplification. The analysis was carried out to check the quality of isolated DNA, either it is amenable to PCR amplification or not. The purity and clean nature of DNA could be confirmed through complete digestion by the restriction enzyme *Eco R1* (Fig. II). All these results indicate that, the isolated DNA was amenable to further processing in cloning experiments as well as DNA fingerprinting for genetic diversity study of the plant. The method described here is therefore rapid, simple and efficient for the isolation of quality DNA from the plant.

CONCLUSION:

Present method yields good-quality, high-molecular-weight DNA that is free of contaminants and colored pigments and can be amenable to PCR amplification and restriction digestion. It is easy, fast and efficient protocol for isolation of quality DNA from *G.superba* for fingerprinting as well as to carry out genetic fidelity testing of the tissue culture raised plants to ensure quality material.

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Table 1. Optimization of DNA extraction from G.superba using different isolation solutions

Isolation solution		**λ260/ λ280**	
		Liquid Nitrogen	Absolute alcohol
without any modification in standard protocol		1.84±0.054	1.42±0.048
	1.50%	1.86±0.026	1.63±0.038
Different concentration of PVP	2.00%	1.91±0.014	1.78±0.032
	2.50%	1.84±0.036	1.68±0.012
Repeated chloroform and iso-amyl alcohol extractions		1.84±0.036	1.68±0.012
Isolation solution		**DNA yield µg/g tissue**	
		Liquid Nitrogen	Absolute alcohol
without any modification in standard protocol		280±8.4	228±3.6
	1.50%	436±10.2	346±9.3
Different concentration of PVP	2.00%	574±8.6	474±14.2
	2.50%	732±21.6	588±34.4
Repeated chloroform and iso-amyl alcohol extractions		732±2.16	588±34.4

± standard deviation

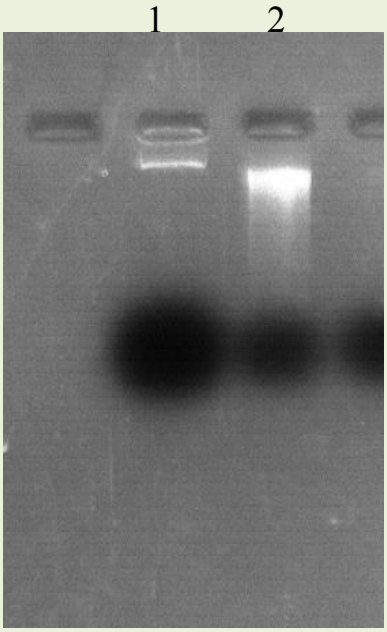

Fig.1. Genomic DNA isolation from leaves of *G. superba* using (1) absolute alcohol and (2) liquid nitrogen

Fig. 2. EcoR1 digested genomic DNA, isolated from leaves of *G. superba* using (1) liquid nitrogen and (2) absolute alcohol

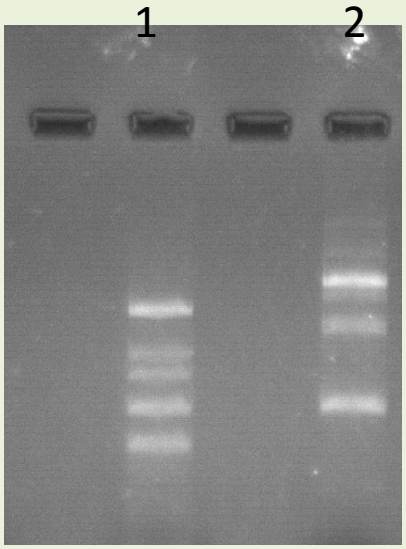

Fig. 3. DNA amplification pattern observed for *G. superba* with species specific primers (lane 1 and 2)