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RESEARCH ARTICLE

INFLUENCE OF FOUR GENOMIC DNA EXTRACTION METHODS ON QUANTITY AND QUALITY OF DNA EXTRACTED FROM *Stevia rebaudiana* BERT

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ABSTRACT

A substantial and low cost DNA isolation protocol is the prerequisite of many molecular biology experiments for the genetic improvement of highly economical medicinal plants. The present study was undertaken to explore the best DNA isolation protocol for *Stevia rebaudiana* Bert., an artificial sweetener. Four methods were evaluated for extraction of DNA namely Kit GeNei™ Genomic DNA extraction (Bangalore GeNei), Kit GeNei™ Plant DNA isolation (Bangalore GeNei), Phenol-Chloroform Method and CTAB method. All four methods evaluated effectively to isolate the DNA from the young and old leaves of *Stevia* plant and produced consistently positive results. The quantity and quality of the DNA extracted was compared using UV-spectrophotometer and agarose gel electrophoresis. It was found that the Kit GeNei™ Plant DNA isolation (Bangalore GeNei) had good quality as well as quantity ($A_{260/280}$, 1.5655) of DNA as compared to other three methods. The present findings will certainly play a vital role in genetic improvement of *Stevia rebaudiana* Bert.

INTRODUCTION

DNA based information is vital in many areas of plant science viz. classification of organisms, species identification using DNA barcoding (Vijayan and Tsou 2010), plant forensics (Craft *et al*., 2007) and plant improvement (Kumar 1999) through molecular breeding. All such applications are successful only after having a good quality DNA isolation protocol because a high molecular mass genomic DNA is the perquisite for PCR (Bryan *et al*., 1998), restriction digestion, southern blot analysis and genomic library construction (Cao *et al*., 2003; Rahman 2007; Md and Rahman 2009). For isolation of quality DNA, many standard protocols are available for a number of plant species and from different parts of the plant. Several publications on plant DNA isolation itself evident that the DNA isolation for every plant needs a new method or modification in the available protocol for optimum results. The unchanged protocol fails to give best results because of the presence of many variable obstacles like polysaccharides, phenolics and other secondary metabolites (Jabbarzadeh *et al*., 2009). The presence of these obstacles often interferes with the isolation of intact DNA (Karaca *et al*., 2005). Economically important medicinal plant *Stevia rebaudiana* Bert., an herbaceous perennial of the composite family is a potent zero calories natural sweetener. *Stevia rebaudiana* is well known for its medicinal properties (Arya *et al*., 2012a; b). Stevioside A, present in the leaves of *Stevia* is 150-200 times sweeter than sucrose. Researchers increased the *Stevia* glycoside concentration in plant leaves up to 20% after an extensive breeding and selection programme (Brandle and Rosa 1992; Rajasekaran *et al*. 2008). Such progress needs a boost to combat the challenge of diabetes and coup with the farmer's demand of superior variety and planting material. So molecular techniques could be an important tool for *Stevia* improvement and for this, isolation of quality DNA is an important prerequisite. Therefore, the present investigation was carried out to compare the commonly available DNA isolation protocols for their efficacy in the isolation of quality DNA.

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MATERIALS AND METHODS

Sample Collections

The samples of *Stevia rebaudiana* Bert. were collected from the micropropagated plants in the Plant Tissue Culture laboratory at Meerut Institute of Engineering and Technology, Meerut, UP, India. The plant materials used for DNA isolation were young leaves (3-7 days old) from tissue-cultured plants and old leaves (35-40 days old) from field transferred plants.

GeNei™ Genomic DNA Extraction (from leaves) (Bangalore GeNei)

DNA from *Stevia rebaudiana* Bert. leaves was extracted using GeNei™ Genomic DNA Extraction (from leaves) (Bangalore GeNei) kit. Leaves were washed with sterile water and rinsed with alcohol. 500µl of cell lysis buffer (pre-cooled) was added to the small *Stevia* leaves discs (young and old) into 1.5 ml micro centrifuge tubes. The mixtures were homogenized to get the lysate and incubated in the water bath at 65°C for 5 min. The samples were again crushed to release more green pigment and further incubated at 70°C for about 60 min. Samples were vortexed followed by spinning at 10,000 rpm for 20 min at room temperature. The supernatant was collected in fresh tube and about 1 ml of alcohol and 50 µl of sodium acetate were added. After mixing, the vials were incubated at -20°C for 60 min and then centrifuged at 12,000 rpm for 30 min at room temperature. The DNA pellet obtained was washed with 70% ethanol for 2 to 3 times. After washing, the DNA pellet was dried at room temperature for 15 min. The DNA pellet was resuspended in 100 µl TE buffer for 1h.

The extracted DNA samples were kept at -20°C to avoid DNA from degradation.

Phenol-Chloroform Method

Phenol-chloroform method by (Brown 1991) was tested with some modifications. Digestion buffer (500 µl) [1% (w/v) SDS 0.8%, Triton X-100, 0.5 M NaCl, 0.1 M Tris-HCl at pH 9, 0.01 M EDTA] were added into 1.5 ml microcentrifuge tube which containing 70 mg leaves and then 40 µl of 10% (w/v) SDS and Proteinase K (20 mg/ml solution) were added. After gentle shaking the tubes were incubated at 55°C for 1h and the mixture was left at room temperature for 20 min. The sample were treated with 500 µl of phenol: chloroform: isoamyl alcohol (25:24:1) and gently the tube were vortexed to homogenize. The sample was left at room temperature for 10 min before doing centrifugation at 13,000 rpm for 5 min. The upper aqueous layer was removed and dispersed in new microcentrifuge tube. The samples were treated with 500 µl of CI (24:1) and were centrifuged at 13,000 rpm for 5 min. The upper aqueous layer was mixed with 1 ml of ice-cold absolute ethanol by rapid inversion of the tubes several times. Then, centrifuged at 6,000 rpm for 30 min and after that the precipitated DNA was collected at the bottom tubes as a white pellet. The pellet was washed with 500 µl of 70% ethanol and was centrifuge at 6,000 rpm for 15 min. The DNA was allowed to dry at room temperature and then resuspended in 100 µl TE buffer (10 mM Tris and 1 mM EDTA, pH 8) for at least 24h at room temperature to fully dissolve before proceeding to the next step. This DNA extraction samples were kept in -20ºC to avoid DNA degradation.

CTAB Method

The DNA was extracted using modified CTAB method (Doyle and Doyle 1987).

GeNei™ Plant DNA Extraction kit (Bangalore GeNei)

The young and old leaves of *Stevia* plant were used for DNA extraction using the GeNei™ Plant DNA Extraction kit (Bangalore GeNei). The kit was provided with the following chemicals: Solution A (CTAB – Extraction solution), solution B (CTAB – NaCl solution), solution C (CTAB – precipitation solution) and solution D (High Salt TE). The prewarm solution A (65°C) + 2 ME was used to crush the plant material. The homogenate was incubated for 60 min at 65°C. After the incubation is over the homogenate was spin at 8000 rpm for 5 min at 4°C. The supernatant was transferred in a fresh vial and one volume of CI in the ratio of 24:1 was added to it and mixed by inverting the vials. 1/10 volume of solution B was added to the supernatant from the previous step and incubated for few minutes at room temperature. Again, the CI was added in equal volume and mixed by inverting the tubes. After mixing tubes were spin at 8000 rpm for 5min at 4°C. The upper aqueous phase is recovered in a fresh vial and 1/10 volume of solution C was added to it. The precipitation thus obtained was collected by spinning the tube at 3000 rpm at 4°C for 5min. After removal of supernatant the pellet was resuspended in high salt TE buffer and kept for incubation at 50°C for 30min. In next step, the DNA was precipitated by adding ice-cold iso-propanol. DNA was pellet out by spinning at 10,000 rpm at 4°C for 15 min. Washing and storage of the DNA was done as per the protocol mentioned earlier.

Measurement of DNA Purity and Quality

The genomic DNA extracted was measured using a UV-spectrophotometer. The purity of DNA was determined by calculating the ratio of absorbance at 260 nm to that of 280 nm. The DNA concentration was determined by the formula referenced by (Linacero *et al*., 1998). For analyzing quality of DNA samples were subjected to 1% (w/v) agarose gel.

RESULTS AND DISCUSSION

Medicinal plants are more demanding in the task of DNA isolation because of the presence of high amount of polysaccharide and phenolics in them. It poses a big challenge to design a best DNA isolation protocol for medicinal plant. For present investigation *Stevia rebaudiana* Bert., a highly economical medicinal plant (Das *et al*., 2011) was selected for testing the efficacy of the commonly used DNA isolation protocols in laboratories. The quantity and quality of extracted genomic DNA from young and old leaves of *Stevia* using the four different methods was investigated. (Table 1) and the agarose gel electrophoresis was also used to test the quality and quantity of isolated genomic DNA (Semagn *et al*., 2006). DNA extracted following the Kit GeNei™ Genomic DNA extraction (Bangalore GeNei) shows that the ratio of OD at 260 and 280nm was 1.231 from young leaves and 1.142 for old leaves of the *Stevia* plant (Table 1). Though the DNA can be extracted either from fresh, lyophilized, preserved or dried samples but for obtaining good quality DNA fresh material is recommended (Zidani, *et al*., 2005; Semagn *et al*., 2006; Srivastava *et al*., 2010). Similar results were observed with Phenol-Chloroform Method (OD $A_{260/280}$: 1.129 for young leaves and 1.013 for old leaves). Results showed that both of these methods yield low quality DNA. Spectrophotometric analysis was also confirmed by the gel electrophoresis as evident by sharp and high intensity bands with Kit GeNei™ Genomic DNA extraction (Bangalore GeNei) as well as a smeared or low intensity bands with other methods (Figure 1and Figure 2). Mature plant samples are hard and the cells have very tough cell wall thus vigorous method use to break cell wall apply excessive force that makes the degradation of very high molecular weight molecules through shearing (Croy *et al*., 1993).

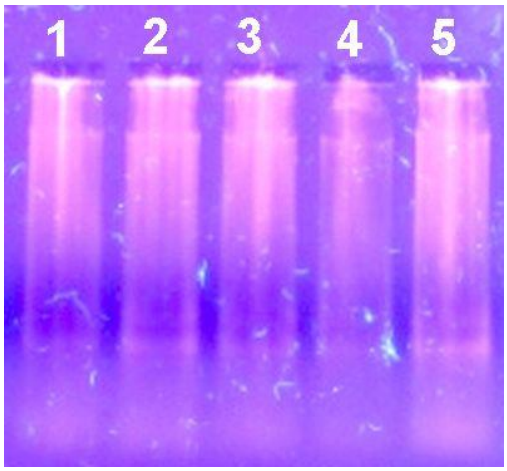

Figure 1. Agarose gel electrophoresis of DNA samples: Lane1and 2: DNA extracted from old and young leaves using Kit GeNei™ Plant DNA isolation (Bangalore GeNei) method; Lane 4and 5: DNA extracted from old and young leaves using CTAB method

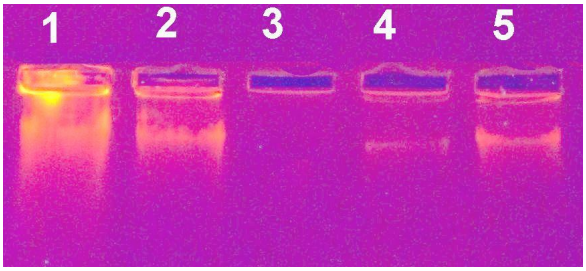

Figure 2. Agarose gel electrophoresis of DNA samples: Lane1, 2 and 3: DNA extracted from old and young leaves using Kit GeNei™ Genomic DNA isolation (Bangalore GeNei) method; Lane 4and 5: DNA extracted from old and young leaves using Phenol Chlorofoam method.

Table 1. Quality and quantity assessment of DNA isolated from *Stevia rebaudiana* Bert. leaves using different methods

Method	Sample	$A_{260/280}$	Total DNA (ng)
Kit GeNei™ Genomic DNA extraction (Bangalore GeNei)	Young leaf	1.231	1364
	Old leaf	1.142	1130
Phenol-Chloroform Method	Young leaf	1.129	973
	Old leaf	1.013	690
CTAB method	Young leaf	1.462	2021
	Old leaf	1.321	1438
Kit GeNei™ Plant DNA isolation (Bangalore GeNei)	Young leaf	1.565	2421
	Old leaf	1.413	2139

CTAB method and Kit GeNei™ Plant DNA isolation (Bangalore GeNei) methods were resulted in good yield of pure DNA. Among all the four methods used to isolate DNA, Kit GeNei™ Plant DNA isolation (Bangalore GeNei) method yield relatively pure DNA from young ($A_{260/280}$: 1.565) as well as old leaves ($A_{260/280}$: 1.413) of *Stevia* as in this method the removal of membranes lipids is facilitated by detergents such as SDS, CTAB. The role of SDS and CTAB in the removal of membranes lipid is also evident by the previous findings and also that the DNA is protected from endogenous nucleases by incorporation of EDTA in the extraction buffer which make complex with magnesium ions, a necessary cofactor for most nucleases (Semagn *et al.* 2006). Gel electrophoresis analysis of isolated DNA from young and old leaves of *Stevia* using four different methods are shown in Figure 1 and Figure 2. This could be attributed to the nature of plant material. Young leaves usually shows a good yield of DNA as compared to old leaves (Zidani, *et al.* 2005). DNA isolated using GeNei™ Genomic DNA extraction (Bangalore GeNei) and Phenol-Chloroform Method (Figure 2) showed low yield of DNA. The appearance of smear also confirms the presence of impurities in isolated DNA. Extraction of DNA using Kit GeNei™ Plant DNA isolation (Bangalore GeNei) and CTAB method (Figure 1) substantiate the importance of these protocols. Sharp and high intense bends indicates that the Kit GeNei™ Plant DNA isolation (Bangalore GeNei) method shows potential for the isolation of high quality DNA from *Stevia* leaves.

Conclusion

The present research on DNA isolation from *Stevia* leaves was attempted using the most common methods available in the laboratories. Our findings suggest that the Kit GeNei™ Plant DNA isolation (Bangalore GeNei) method is the best method as compared to other methods of DNA isolation. CTAB method was also effective; however, further modification in the existing protocol and use of protein and RNA degrading enzymes may result in the better quality of DNA that can directly be used for cloning and PCR.

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