Standardization of extraction of genomic DNA and PCR-RFLP conditions of *Allium stracheyi*: A high altitude plant

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Abstract

DNA extraction is difficult in many plants because of metabolites that interfere with DNA isolation procedures and subsequent applications such as DNA restriction, amplification and cloning. We developed a simple, rapid and efficient method for isolating genomic DNA from seeds of *Allium stracheyi* that is free from polysaccharides and polyphenols. This newly developed protocol include the use of 2.5 M NaCl, 3% polyvinylpyrrolidone (PVP), 3% mercaptoethanol, 0.15% sodium sulfite, and 80% ethanol in the extraction as well as reducing the centrifugation times during the separation and precipitation of the DNA. Isolated genomic DNA showed high purity and high quantity. The purity of isolated genomic DNA was confirmed by biophotometric analyses ($A_{260/280}$ of 1.840). [Academia Arena, 2010;2(7):11-14] (ISSN 1553-992X).

Key words: DNA isolation, *Allium stracheyi,* Secondary metabolites, Seeds & Polyphenols.

Introduction

Allium stracheyi (Jambu) is a perennial herb, flowers are white in color about 35-40 centimeters in height and is traditionally being used by the local people as spice for flavoring. It is mainly found at the height of 2500-3000 meters of Alpine Himalayas of Uttarakhand, India near moist rock, dry rock and steep slope with a strong preference of sunny site. Edible plant part used includes flowers, leaves, root and bulb. In the genetic improvement process, it is desirable to use molecular markers for screening of accessions, choosing of parents and selection of progeny. The presence of certain metabolites can hamper the DNA isolation procedures and reactions such as DNA restriction, amplification and cloning. Problems encountered in the isolation and purification of high molecular weight genomic DNA from seeds comprise: co-isolation of highly viscous polysaccharides, and inhibitor compounds like polyphenols and other secondary metabolites which directly or indirectly interfere with the further enzymatic reactions (Weishing *et al*., 1995). The presence of polyphenols, which are powerful oxidising agents present in many plant species, can reduce the yield and purity of extracted DNA (Porebski *et al*., 1997). The use of β-mercaptoethanol, ascorbic acid, bovine serum albumin (BSA), sodium azide, and polyvinylpyrrolidone (PVP) (Dawson and Magee, 1995; Clark, 1997). Phenol extractions coupled with SDS are also helpful. However, SDS-phenol tends to produce low DNA yields of plants rich in polyphenolics.

Plant species belonging to the same or related genera can exhibit enormous variability in the complexity of pathways of dispensable functions. Thus, the biochemical composition in plant tissues of different species is expected to vary considerably. The chemotypic heterogeneity among species may not permit optimal DNA yields from one isolation protocol, and perhaps even closely related species may require different isolation protocols (Weishing *et al*., 1995). Most of the protocols recommend isolation of DNA from fresh tissues, but it is also required to isolate DNA from seeds. These situations imposed the development of the protocols for isolating DNA from different plant organs, including dry tissues and seeds. In addition to this, from the previously recommended protocols we could not recover a reasonable amount of purified, digestible genomic DNA. The seeds of *Allium stracheyi* are considered to be difficult for DNA isolation due to its high polyphenolic content and cold temperature at high altitude (3000 m). Here we described a rapid DNA isolation protocol that can be used for isolation of DNA from leaves of *Allium stracheyi* collected from thirteen different landraces varied in their altitudes of Uttarakhand, India. However, to the best of our knowledge, no such studies have been performed previously on *Allium stracheyi* seeds of high altitude.

Materials and Methods

We performed several experiments before finding one that yielded a good amount of genomic DNA from seeds of *Allium stracheyi*. A number of experiments were set up to close down the polysaccharide contamination of the DNA by using sodium chloride (concentrations 1.0- 3.0 M) and polyphenols. For removing the polyphenol content from the isolated genomic DNA we used β-mercaptoethanol (2-5%), PVP (2-6%) and sodium sulfite (0.06-0.16%). An optimized protocol was design on the basis of results obtained from the above experiments.

Plant materials for DNA isolation

The seeds of *Allium stracheyi,* which were collected from thirteen different altitudes of Uttarakhand, India, during October-November 2009. Immediately after collection, the leaves and seed were stored at 4ºC till the start of experiments. Before extraction, the seeds were washed with autoclaved distilled water. Specimens of seed samples were submitted in the National Bureau of Plant Genetic Resources (NBPGR), Pusa Campus, New Delhi. The taxonomic identification of these specimens was performed and the accession no. is IC-56745.

Reagents and chemicals

- Extraction buffer: 100 mM Tris-HCl (pH 8.0), 25 mM EDTA, 2.5 M NaCl, 2.5% CTAB, 3.0 % β-mercaptoethanol (v/v) (added immediately before use) and 3% PVP (w/v) (added immediately before use)), 0.15% sodium sulfite.
- High salt TE buffer: 1 M NaCl, 10 mM Tris-Cl (pH 8.0) and 1 mM EDTA.
- TE buffer : 10 mM Tris-HCl (pH 8) and 1 mM EDTA (pH 8).
- Enzymes: Alu I and *Taq* DNA Polymerase (Genei, India).
- Buffers: Alu I buffer and *Taq* DNApolymerase buffer (Genei, India).
- Nucleotides: dNTPs (G, A, T, C) and primers (Genei, India).

DNA isolation and purification

Five hundred mg of seed samples was weighed and placed on a precooled mortar. Liquid nitrogen was poured onto the sample and allowed to evaporate completely. The seeds were macerated into fine powder with a pestle and added to 5 ml of preheated (60°C) extraction buffer. The mixture was incubated for 1 ½ h at 60°C, with constant shaking at intervals followed by cooling at room temperature (RT) with gentle shaking. An equal volume of chloroform-isoamylalcohol (24:1) was added to the mixture. The tubes were mixed gently at RT for 15 min to produce a uniform emulsion. The emulsion was centrifuged at 10, 000 rpm for 15 min at 4°C.

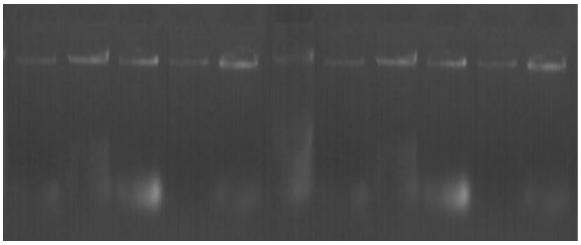

Fig. 1: Showing Bands of genomic DNA

The upper aqueous layer was separated and collected into a fresh tube. A second chloroform-isoamylalcohol extraction was performed. The supernatant was carefully decanted and transferred to a new tube and then precipitated with chilled 2/3 volume of isopropanol. The precipitated nucleic acids were collected and washed twice with the buffer (80% ethanol; 10 mM ammonium acetate; TE: 1 mM Tris and 0.1 mM EDTA [pH 8]). The pellets were washed twice with 70% ethanol followed by centrifugation at 4ºC and 10,000 rpm for 10 min. Finally, pellets were air dried and resuspended in TE buffer. The dissolved nucleic acids were brought to 2.5 M NaCl and reprecipitated by using 2 vol of 70% ethanol. The pellets were washed twice with 80% ethanol, dried, and resuspended in 100 µL of TE buffer. The tube was incubated at 40°C for 10 min to dissolve genomic DNA, and RNase treatment was followed.

Quantification of Genomic DNA

The DNA yield per microgram of seed samples was measured by using a Biophotometer (Eppenndorf) at 260 nm. DNA purity was determined by calculating the absorbance ratio A260/280. Pure DNA has a ratio of 1.8 ± 0.1 (Clark, 1997). The DNA sample was also quantified on 0.8% agarose gel electrophoresis.

PCR amplification

Polymerase chain reactions (PCRs) for amplification of DNA preparations were carried out in a 25 µl reaction volume. A reaction tube contained 100 ng of template DNA, 1× PCR buffer, 1 units of *Taq* DNA polymerase, 50mM each of dNTPs, 3 mM $MgCl_2$ and 5 pmol of decanucleotide primers. The amplifications were carried out using PCR thermal cycler (Eppendrof, Germany). Initial denaturation was for 3 min at 94ºC followed by 35 cycles of 3 min at 94ºC, 2 min at 54ºC, 3 min at 72 ºC, and a 10 min final extension step at 72ºC. The success each PCR reaction was verified by electrophoresis. The

amplified products were loaded in a 1.2% agarose gel containing 5 mg ml−1 of ethidium bromide. Custom decanucleotide primers were synthesised from M/S Bangalore Genie, India. The primer had the following sequences:

Forward primer
5'- CGAAATCGGTAGACGCTACG - 3'
Reverse primer
5'- GGGGATAGGGACTTGAACGG- 3'

Results and Discussion

The plants that are sources of natural products or bio-active substances also produce large amounts of secondary metabolites and valuable for human being. Thus, while working with a seed samples enriched with secondary metabolites it is common to encounter problems arising from the polysaccharides, polyphenols and other secondary metabolites in the lysate and the DNA preparations. The secondary compounds may hamper DNA isolation as well as any further reaction to be carried out on DNA preparations for example; restriction enzymes may be inhibited because of the presence of unusual substances.

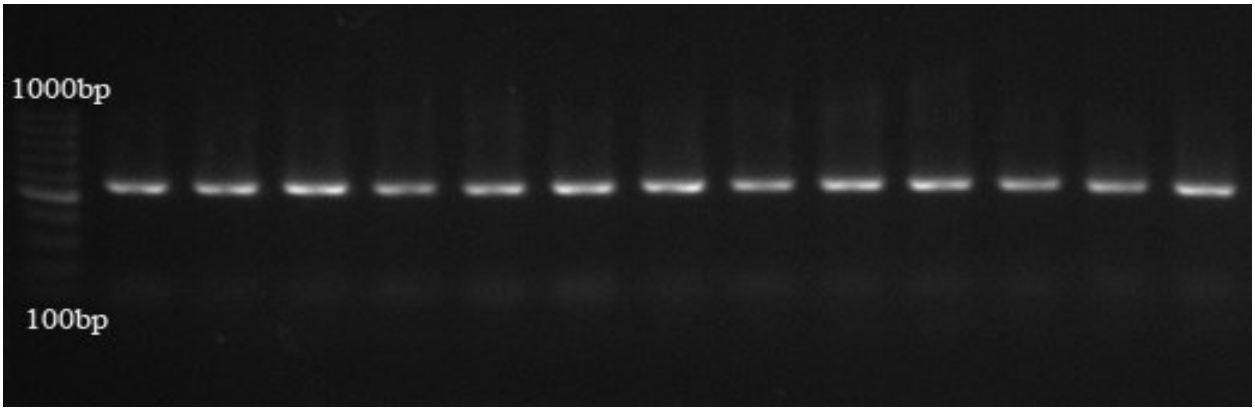

Fig 2: Showing amplified bands from seeds of *Allium stracheyi* using PCR-RFLP primer

In our experiments we encountered difficulties from the stage of cell lysis to DNA separation in the supernatant and subsequent reactions when following the procedures described by Doyle and Doyle (1990), Murray and Thompson (1980), Dellaporta *et al*. (1983) and Porebski *et al*. (1997). Major problems encountered were low DNA yield or poor PCR amplification reactions and restriction endonuclease digestion. Our protocol involves several modifications with one parameter tested at a time to address the problem of phenolics. Modifications included use of different concentrations of β-mercaptoethanol (2-5%), use of PVP at different concentrations (2-6%), and use of sodium sulfite (0.06%, 0.17%). Use of 4% β-mercaptoethanol, 3% PVP and 0.15% sodium sulfite was found to be most appropriate.

Highly purified genomic DNA was obtained when the optimised protocol described in "Materials and Methods" was used. A sufficient amount of clean genomic DNA was obtained with this method (Figure 1). The yield was ranged from 20-50 µg per gram of seed material collected from different locations. The A260/230 nm ratio was 1.826, while the A260/280 nm ratio was 1.840. Our protocol involves isopropanol precipitation of DNA initially at room temperature. Moreover, the procedure also eliminates the necessity of phenol, which makes the method less hazardous. Further, the addition of high concentration of PVP and β-mercaptoethanol were helpful in removing the polyphenols from the seeds of *Allium stracheyi*. The problem arising from the presence of high levels of polysaccharides was overcome by using NaCl at a higher concentration. The use of sodium sulfite is also recommended to prevent oxidation (Aljanabi *et al.*, 1999), it was included in the extraction buffer but at a (Mr 10000), DNA free from phenolics. The amplified product is having molecular weight of around 515 base pair to 605 base pair.

This protocol provides good quality of genomic DNA from the seeds of *Allium stracheyi*, which was not, reported earlier. The secondary metabolites, when isolated along with the DNA, do inhibit PCR amplification and restriction digestion.

This indicated that the isolated DNA was amenable to further processing in cloning experiments as well as DNA finger printing. The method described here for the extraction of genomic DNA will be useful for molecular, genetic diversity, and transgenic studies in *Allium stracheyi.* The utility of the isolated DNA for use in DNA finger printing

was demonstrated with several random primers. Thus the method is simple rapid and less hazardous.

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