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DNA Extraction from Moth bean mutants containing high polyphenols and polysaccharides

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ABSTRACT

A number of methods are available for isolation of plant genomic DNA, but in these procedures due to variability in plant tissue composition (like polyphenols, tannins and polysaccharides) practical difficulties are always met. With consistent isolation of quality DNA from Moth bean (Vigna aconitifolia (Jacq.) Marachel) is particularly problematical due to the presence of phenolic compounds. Inconsistencies in extraction result can be attributed to the growth stages of the plant material analyzed. The mature moth bean leaves which contain high levels of polyphenols, tannins and polysaccharides, were used as a study group. The method involves a modified CTAB extraction employing high salt concentration to remove polysaccharides, the use of polyvinyl pyrrolidone (PVP) to remove polyphenols, an RNase treatment and a phenol-chloroform and chloroform-isoamyl alcohol extraction procedure. The results from the local Moth bean-88 and its seven mutants were consistently amplifiable in RAPD reaction with as little as l µg DNA/25ul reaction.

Keywords: Moth bean, mutants, DNA extraction, PCR and RAPD.

INTRODUCTION

A large number of legume species hitherto unexploited possess great potential for contributing to not only protein rich food for human but also excellent quality forage for animals. Among such novel legumes, the moth bean *(Vigna aconitifolia* (Jacq.) Marachel) is quite notable. Although the moth bean plant has immense potential in agriculture, it has failed to gain attention of farmers of our country. This has happened due to some shortcomings possessed by the moth bean plant. Some such shortcomings comprise: semiviny, indeterminate plant habit creating problems for mechanical harvesting and the presence of antinutritional factors like trypsin inhibitors, polyphenols, tannins and lectins in its seeds. In view of this background, the utility of novel approaches has been thought quite crucial for developing

dwarf/erect, early maturing, high yielding and anti-nutritional component free genotypes of moth bean. Keeping in mind the excellent potential of induced mutation in plant improvement programmes, it was thought very much appropriate and relevant to initiate the mutation breeding work in Moth bean for achieving its genetic improvement

The genetic variability may lead to the improvement of Moth bean crop. For checking the variability, it was considered desirable, first to isolate the plant genomic DNA. The current DNA isolation methods have limitations in regards to their abilities to obtain good quality/quantity of DNA from Moth bean plant due to the presence of high polyphenols, lectins and polysaccharides in the latter. RAPD is a common molecular approach employed in DNA fingerprinting analysis for noting genetic differentiation in molecular taxonomy and other allied applications (Lin et al., 1996). Another prominent objective of the present work has been to ascertain the genetic variability induced through mutation in moth bean by the RAPD approach. Isolating pure and amplifiable high-molecular weight genomic DNA from the Moth bean induced mutants has always been found crucial bottleneck because it contains high levels of tannins, lectins, polyphenols and other polysaccharide compounds. Several methods of DNA isolation are coming available besides, variant of a few principal ones (Dellaprta et al., 1985; Doyle and Doyle 1987). Unlike non-plant DNA isolation protocols, methods used to be adjusted to each species and even to each tissue because of the wide range of primary and secondary metabolites available there in (Sangwan et al., 1998). The current methods produce degraded and denatured DNA or give extremely poor yields. We were uniformly unsuccessful in our attempts to amplify Moth bean DNA by PCR using other recommended methods, like Dellaprta et al. (1983); Doyle and Doyle (1987), Porebski et al., (1997) and Sharma and Anjaiah (2000). On account of this background, we considered it necessary to devise a protocol for DNA extraction from Moth bean mutant plants which would yield DNA suitable for PCR, especially for RAPD analysis.

MATERIAL AND METHOD

Plant materials:

Fresh leaves of Moth bean mutants as well as controlled plants were harvested from the research field. These leaves were brought to laboratory in an icebox and stored at -20° C until further analyses were carried out.

Solutions and Reagents:

• CTAB (20% [W/V]), 1M Tris-HCl (Ph-8), 5M EDTA (Ph-8), 5M NaCl, 3M Sodium acetate, ethanol (AR grade), Chloroform-IAA (24:1), Polyphenol pyrrolidone (PVP) (40,000 mol. wt.) 2-p-mercaptoethanol.

• Extraction Buffer: 100 mM Tris-HCl (pH-8), 20mM EDTA (pH-8), 1.4 M NaCl, CTAB (20%[w/v]), 2-p-mercaptoethanol (0.3%[v/vj) (added to buffer just before use), l00mg PVP per gram of tissue (added in the mortar while crushing the tissue).

- T.E. Buffer: 10 mM Tris-HCl, 1 mM EDTA (Ph-8.4)
- RNaseA: l0mg/ml.
- Proteinase-K: 1mg/ml.

Seven Moth bean induced mutants with control plants were used. The modification of CTAB extraction procedure of Doyle and Doyle (1990) includes high NaCl concentrations in the buffer to remove polysaccharides and polyvinyl pyrrolidone (PVP) to remove polyphenols (Maliyakal, 1992). An extended one-hour RNase treatment was required for the DNA product to become RNA free and PCR amplifiable, with an additional phenol chloroform step (Saghai-Maroof et al., 1984) for removing any excess protein left. These steps provided the principal key to success for collecting DNA from the leaves of Moth bean.

DNA Extraction protocol:

- 0.5g of leaves were ground by using mortar and pestle in the presence of liquid nitrogen with PVP, until finely ground.
- The frozen ground leaf tissue was transformed to 20ml polypropylene centrifuge tubes.
- 5ml of extraction buffer (containing 0.3% [v/v] p-mercaptoethanol) was added, so as to adjust the pH up to 7.5-8.
- Incubated at 65 °C in the water bath for 2 hours with intermittent shaking and for pH monitoring.
- Removed from the heat and cooled to room temperature for 4 to 5 minutes.
- Added 6ml of chloroform-IAA (24:1) and mixed by inversion to form an emulsion.
- After mixing thoroughly, spin at 5000 rpm for 10 minutes at 4°C.
- Transferred the top aqueous solution to new tubes using wide bore pipette tip.
- Repeated chloroform-IAA extraction to remove cloudiness (PVP) in aqueous phase.
- Added half volume of 5M NaCl to the final aqueous

solution recovered. Mixed well. Added two volumes of cold (-20°C) 95% ethanol Mixed by inversion. If required, placed in freezer (-20°C) for 10 minutes to accentuate precipitation. The solution was left at 4 to 6°C to precipitate overnight.

- Centrifuged at 3000 rpm for 6 minutes.
- Removed the supernatant and wash pellet with cold (0-4°C) 70% v/v ethanol.
- Dried pellets in 37°C oven or vacuum until dried it well (~1 hour).
- Dissolved in 300ul of T.E. buffer for overnight at 4 to 6°C. Transferred it to 1.5ml Eppendorf tubes.
- Added 4ul of RNase-A (l0 mg/ml) and incubated in 37°C water bath for approximately 1 hour. Added 3ul of proteinase-K (1 mg/ml) and incubated at 3 7°C for half an hour.
- Added an equal volume of phenol-chloroform (1:1) to each Eppendorf tube.
- Vortexed briefly and spun (in microfuge) at 2000 rpm for 15 minutes. Collected upper layer in new 1.5ml tubes. Added 50ul T.E. buffer to phenol phase.
- Vortexed, spun, remove upper layer and added to sample.
- Precipitated DNA from aqueous layer by adding 1/10th volume of 3M sodium acetate and chilled ethanol at 4°C.
- Incubated at -20°C for overnight.
- Centrifuged at 12000 rpm for 15 minutes. Removed the supernatant and washed the pellet with 70% v/v ethanol. Ethanol was removed.
- Dissolved the pillet in T.E. buffer after drying in speed vacuum (Savant) allowed time to complete resuspension.
- DNA concentrations were measured by running aliquotes on a 0.5% (w/v) agarose gel and by taking the absorbance at 260nm (Sambrook et al., 2001).

PCR amplification:

PCR for amplifying the DNA preparations was carried out in a 25 ul volume of reaction mixture. A reaction tube contained 25ng of DN, 0.6ul of Taq DNA polymerase enzyme, 100 mM of each, dNTPs, 1xTaq DNA polymerase buffer and 10 P mol. deca nucleotide primers. Amplifications were carried out by using a DNA Thermal Cycler (Eppendorf, Mastercycler gradient) with the following parameters: 94°C for 5 min: 45 cycles at 94°C for 1 min, 36°C for 1 min and 72°C for 2 min and a final extension at 72°C for 5 min. PCR products were subjected to agarose gel (0.8% [w/v]) electrophoresis in 0.5x TBE buffer, along with 100-bp DNA ladders (Bangalore Genei, India) as size markers.. DNA was stained with ethidium bromide and photographed under UV light.

RESULT AND DISCUSSION

The method described here can be used to obtain consistently high quality genomic DNA from Moth bean. Although large quantities of DNA could be obtained by Dellaporta method, but the obtained DNA contaminating always revealed with high levels of polysaccharides and polyphenols making it opaque and difficult to dissolve. By using the modified protocol as above, good quality DNA could be recovered and satisfactorily used in RAPD and PCR application. The results indicate the total genomic DNA isolated by this protocol besides the purity of DNA. PCR analysis of Moth bean mutants as indicated by ethidium bromide staining and RAPD revealed that the PCR was successfully run by using quantities as small as 0.5 ng in 25µl reaction. When amount was increased beyond 5 ng. DNA bands became less distinct. Although such DNA yields were large enough to permit as many as 100 PCR reactions or good number of RAPD reactions.

Table: 1. Isolated DNA yield obtained from different viable mutants of Moth Bean by modified DNA extraction protocol.

Sr. No.	Mutants	Optical Density X260	Optical Density X280	X260 / X280	Concentration of DNA ug/ul	DNA Yield ug/gm tissues
1.	Control	0.0880	0.0451	1.950	4.40	440
2	Branched	0.0930	0.0500	1.860	4.65	465
3	Tall	0.0875	0.0477	1.832	4.35	435
4	EM	0.0887	0.0506	1.752	4.43	443
5	LM	0.0795	0.0396	2.006	3.97	397
6	DW&EH	0.1079	0.0614	1.755	5.39	539
7	HY	0.0758	0.0404	1.875	3.80	380
8	SP/BS	0.1041	0.0276	1.93	5.20	520

CONCLUSION

The highest yield of *Pleurotus sajorkaju* on soyabean straw indicated wide scope for mushroom cultivation. This can also be considered as an agribusiness for the people of this region to improve their financial status and health. Mushroom cultivation converts energy stored in the straw and provide protein rich foods for human and animals. The agro waste thus can be used more efficiently instead of burning of them to generate heat energy also may cause air pollution. The Present study explored the possibilities of cultivating Pleurotus sajor kaju on different agro waste. Soyabean is one of the major cash crop of this region was found to be the most suitable agro waste for oyster mushroom cultivation.

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Conflicts of Interest: The authors declare no conflict of interest.

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