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A simple modified method of DNA extraction from seeds for PCR amplifications*

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The field grow-out test (GOT) is the common method of ascertaining genetic purity of a seed lot, where morphological characteristics are scored at various stages of plant growth. However, the GOT are tedious, time consuming and require large areas. Therefore, alternative reliable methods to assess genetic purity of seed sample are needed to help seed producers to assure high quality standards. Biochemical markers like isoenzymes and seed proteins have been suggested for seed purity determination but these techniques have low discrimination power except in some cases, where IEF is recommended for testing genetic purity of hybrids and varieties (ISTA 2004). Restriction fragment length polymorphism (RFLP) has also been used for this purpose, but being expensive and tedious, these are not easily adaptable in routine testing of a large number of commercial seed lots. Polymerase chain reaction (PCR) based techniques have emerged as potential and user-friendly methods which are fast, reliable and require minimal amount of DNA (usually up to 10 kb), for investigation. PCR techniques such as random amplified polymorphic DNA (RAPD, Williams *et al.* 1990), inter-simple sequence repeat (ISSR; Zietkiewicz *et al.* 1994), sequence tagged microsatelite site (STMS, Beckmann and Soller 1990), sequence characterized amplified region (SCAR, McDermott *et al.* 1994) and amplified fragment length polymorphism (AFLP, Vos *et al.* 1995) have been used in plants for assessment of genetic purity, identification of genotypes associated with genes of interest, molecular characterization of plant varieties, mapping and genetic diversity studies. A simple and rapid DNA extraction method is needed for studies such as genetic purity testing of hybrid seeds. Several methods for minimizing the DNA extraction steps have been reported (Berthomieu and Meyer 1991, Edwards *et al.* 1991, Horne *et al.* 2004), but these require grinding of a large amount of plant tissues in liquid nitrogen. Timely availability of liquid nitrogen is limited in many of the developing countries. In addition, growth and management of plants and storage of the plant samples in freezers below–20°C is often difficult due to availability of uninterrupted power supply, space and other infra-structure constraints. Kang *et al*. (1998) has reported a rapid DNA isolation protocol, but the same was not found suitable when large amount of DNA was required (Mishra *et al.* 2008). Moreover, use of proteinase K in this protocol was not found suitable, besides increasing the cost. Also, they used liquid nitrogen for isolation of DNA, which again is a limiting factor for many laboratories. We, therefore, propose a simple, efficient and rapid protocol for isolation of DNA from seeds which could be useful for molecular studies based on PCR amplification.

The experiment was conducted at Genetic Purity Laboratory of Division of Seed Science and Technology, Indian Agricultural Research Institute, New Delhi during 2010. Seeds of seven genotypes of Indian mustard, one variety each of rice, wheat and cotton were hydrated overnight. The imbibed seed (50mg) were ground in 400 µl of extraction buffer (200 mM Tris-HCl, *p*H 8.0; 200 mM NaCl; 25 mM EDTA and 0.5% SDS). In case of cotton the seed coat was removed before grinding. To this 400 µl of CTAB solution (2% CTAB; 100 mM Tris- HCl, *p*H 8.0; 20 mM EDTA, pH 8.0; 1.4M NaCl and 1% PVP were added. In place of using 1% PVP, 2% PVP was used for cotton and Indian mustard in the above CTAB solution. The tubes were incubated at 65ºC for 1 hr with intermittent shaking and swirling at 25 min. intervals and cooled to room temperature. Equal volumes of phenol:chloroform:isoamyl alcohol (25:24:1)was added the mixture was centrifuged at 12,000 rpm in a micro centrifuge at 4ºC for 30 min. The supernatant were transferred to new tubes. To this 2/3 volume of chilled isopropanol was added and the tubes were incubated at 4º C for overnight to precipitate the DNA. The contents were once again centrifuged at 12 000 rpm for 5 min. and the supernatant was

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removed. The DNA pellet was washed with 70% ethanol, air dried, and resuspend in 100 µl of TE buffer.

The extracted DNA was quantified by using Bio-photometer (Eppendorff) at A_{230}nm, A_{260} nm and A_{280} nm. The purity of DNA was determined by calculating the ratio of absorbance at 260/280 nm and DNA concentration was calculated in ng/ µL using the in-built software programme of Bio-photometer. PCR analysis of five RAPD primers, viz OPD 12, OPB 08, OPB 17, OPB 18 and OPB 20 was used for testing the diversity of seven Indian mustard (varieties Pusa Jaikisan, Kranti, Pusa Purak, Varuna, Pusa Mahak, Pusa Bold and Pusa Tarak)**,** whereas one variety each of rice (Punjabia*)*, wheat (HD 2987) and cotton (Nath Baba) was used to test the quality of PCR amplification using random primers. The statistical analysis (ANOVA) for the above parameters was carried out using complete random design (CRD).

A 25-µl reaction cocktail was prepared as follows: 10× 2.5 µl*Taq* buffer, 1.0 µl dNTPs (10 mM), 1.0 µl $MgCl_2$ (25 mM), 1.67 µl primer (5 mM), 0.7 µl *Taq* polymerase (5 unit), 16.13 µl sterilized water, and 2.0 µl template DNA (100 ng/ µl). A total of 5 RAPD primers were used to check the amplification with different DNA sample. The thermo cycler was programmed as follows: 2 min. at 95°C; 2 cycles of 30 s at 95°C, 1 min at 37°C, and 2 min. at 72°C; 2 cycles of 30 s at 95°C, 1 min at 35°C, and 2 min. at 72°C; 41 cycles of 30 s at 94°C, 1 min. at 35°C, and 2 min. at 72°C, and a final 5 min. extension at 72°C, followed by cooling down to 4°C. The polymerase chain reaction products (25 µl) were mixed with 6× gel loading buffer (4 µl) and loaded onto an agarose (1.5%, w/v) gel in 1X TBE (Tris-borate-EDTA) buffer mixed with 4 µl ethidium bromide(1µg/µl), and electrophoresis was performed at 80 V for 150 min. The bands were visualized under UV in a Bio Doc Image Analysis System.

High quality DNA free from polyphenols and polysaccharides from the wheat, rice, cotton and Indian mustard seeds were obtained. When spooled out of solution, the DNA is clear or white; there was no visible coloration. The $A_{260/A280}$ ratio of the DNA ranged from 1.65 to 1.91 indicating the isolated DNA is free from protein and RNA contamination. However, yields of the DNA varied with the cultivars in Indian mustard, rice, wheat and cotton (Table 1). In order to check the efficiency and reliability of the method, we amplified the seed DNA of varieties of Indian mustard, rice, wheat and cotton using five RAPD primers. The PCR amplification was robust (Fig 1). Genotyping by PCR analysis of half seed DNA from wheat and rice was reported (Kang *et al.* 1998). They tested direct PCR amplification after a boiling step combined with addition of proteinase K or a chelate resin in the PCR buffer. We have tested our method on the imbibed seeds of plant species, viz wheat, rice, cotton and Indian mustard without adding proteinase K. Using this modified method, we obtained DNA yields of 825.2–3811.3ng/µl seed depending on seed varieties. The protocol is both quick (200–300 samples can be extracted in 24 hr) and economic (US$ 15/ 100 samples), and well suited for routine analysis of a large number of samples by the technicians in seed testing laboratories. This simple protocol also avoids dependence on ready-to-use DNA extraction kits, supplied by a number of manufacturers which cost on an average of US$ 80/100 samples.

In conclusion, the above protocol for DNA isolation from seed is both easy and reliable. The amount and quality of the DNA is suitable for PCR marker studies. The protocol could be scaled up or down depending on the quantity of the seeds available as well as the requirement of DNA material for a particular molecular analysis. This method will be useful for molecular diagnostic and could also be extended to extract DNA from the seeds of other plant species as well.

Table 1 Quality and quantity of DNA extracted from seed of wheat, rice, cotton and Indian mustard

Crop/variety	OD_{260}	OD_{280}	OD_{230}	$OD_{260/280}$	$OD_{260/230}$	Concentration	
						(ng/µl)	ng/mg of seed
Wheat (HD- 2987)	1.610	0.915	1.014	1.76	1.59	3 811.3	76.22
Rice (Punjabia)	0.489	0.280	0.183	1.74	2.68	2 300.6	46.01
Cotton (Nath Baba)	1.10	0.658	0.593	1.67	1.85	2 720.8	54.41
Indian mustard							
Pusa. Jaikisan	0.915	0.525	0.782	1.74	1.17	2 107.0	42.14
Kranti	0.020	0.012	0.015	1.65	1.37	825.2	16.50
Pusa Purak	0.525	0.317	0.325	1.66	1.61	2 193.0	43.86
Pusa Mahak	0.711	0.401	0.605	1.77	1.17	866.4	37.32
Pusa Bold	0.232	0.140	0.162	1.66	1.44	923.6	18.47
Pusa Tarak	0.238	0.141	0.188	1.69	1.26	1 011.4	20.22
Varuna	0.024	0.012	0.014	1.91	1.61	1 130.3	22.60
Mean	0.586	0.340	0.388	1.725	1.574	1 888.9	37.77
CV (%)	84.12	82.95	85.01	4.484	27.37	48.47	48.48
CD (*P*=0.05)	0.061	0.019	0.003	0.026	0.026	1.129	0.032

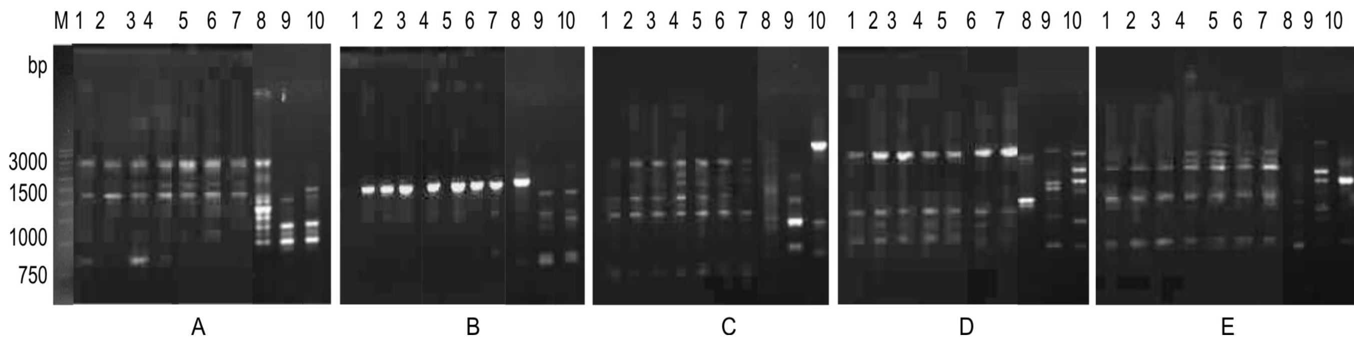

Fig 1 PCR amplification of DNA extracted from seeds of seven Indian mustard varieties, one variety each of rice, wheat and cotton using five RAPD primers. The DNA extraction was done using seeds of each variety and about 100 ng of DNA was used for the PCR reaction. Amplified PCR products were electrophoresed on an agarose gel (1.5%). M, marker; Lane 1, Pusa Jaikisan; lane 2, Kranti; lane 3, Pusa Purak; lane 4, Pusa Mahak; lane 5, Pusa Bold; lane 6, Pusa Tarak; lane 7, Varuna; lane 8, Wheat (HD 2987); lane 9, Rice (Punjabia) and lane10, Cotton (Nath Baba). (A-E: Primers, viz OPD 12, OPB 08, OPB 17, OPB 18 and OPB 20).

SUMMARY

Rapid and reliable protocols for testing genetic purity of seed are an important requirement for quality assurance. This method was applied to Indian mustard, rice, wheat and cotton. Yield and quality of DNA was good for PCR analysis. DNA yield ranged from 16.4 to 76.22 ng/mg seed depending on plant species and varieties. The $A_{260/280}$ ratio ranged from 1.65-1.91, indicating that the isolated DNA was fairly clean and free from protein and RNA contaminations. By adopting this method nearly 200–300 sample can be extracted in 24 hr, which will be useful for assessment of genetic purity of seed lots during seed multiplication as well as in breeding programmes that requires rapid screening of large population.

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