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A SIMPLE AND RAPID DNA ISOLATION METHOD FROM DRY PEA SEEDS SUITABLE FOR PCR ANALYSES

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ABSTRACT

Pea (*Pisum sativum* L.) is an important legume grown and consumed extensively worldwide. As a rich source of proteins, carbohydrates and vitamins, peas are important in human nutrition.

In this study, we compared four different DNA isolation methods from four pea cultivars (F95-927, Specter, Windham and Nicoleta), with some modifications to the original extraction protocols: one SDS extraction buffer without PVP and one SDS extraction buffer with 1% PVP; one CTAB extraction buffer without PVP and one with 1% (1.5%) PVP.

For all four extraction methods we used the same quantity of plant material (0.05 g) and an equal quantity of extraction buffer, so the comparison between methods to be very accurate.

To establish which is the most efficient extraction method, after DNA isolation and purification, we submitted our samples to PCR analyzes with two markers: ISSR marker 17899A and SSR marker AA175.

In my study, based on spectrophotometric measurements and PCR results, I concluded that both CTAB extraction methods were not suitable for DNA extraction from dry pea seeds because they did not show amplification products. The most appropriate DNA extraction method was SDS1 which provided a good quality DNA.

Key words: pea seed, SDS, CTAB, isolation, DNA.

INTRODUCTION

Molecular techniques require isolation of good quality genomic DNA. To extract and purify DNA from seeds is difficult because of the occurrence of diverse compounds that can affect the quality and quantity of DNA, which often make the sample non-amplifiable.

One of the most frequent contaminant in DNA extraction from seeds are polysaccharides; they can make DNA pellets slimy and difficult to handle. Other problematic contaminants are: proteins and DNA polymerase inhibitors like tannins, alkaloids, and polyphenols, which can be inhibitory to additional analysis of the DNA, restriction analysis, etc.

There are several well known extraction methods (Dellaporta et al., 1983; Doyle, 1991, Mogg et al., 2003; Mohammadi et al., 2012) that can be used for a successful DNA extraction:

- cetyl trimethylammonium bromide (CTAB) and its modifications;
- sodium dodecyl sulphate (SDS) and its modifications;
- commercial extraction kits (the main problem with these kits is the high cost per sample).

However, plants belonging to the same or related species can exhibit enormous variability therefore, an improved extraction protocol for isolating pure and intact DNA, suitable for dry pea seeds, could be useful.

MATERIAL AND METHODS

Materials

Seeds were obtained from Pea Breeding Laboratory, NARDI Fundulea, Romania, and consisted of four *Pisum sativum* L. cultivars: F95-927, Specter, Windham and Nicoleta.

The probes where grounded to a fine powder, using a mortar and a pestle.

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In previous attempts, a higher amount of sample (0.07-0.15 grams/probe) was used but the results were not satisfying. In our final tests we concluded that smaller amount, 0.05 g/probe is enough to isolate and extract good quality DNA. The reason to use a smaller amount from each probe is not just less biological material, but also less extraction buffer, so smaller expenses/probe.

Methods

Four methods for DNA isolation were tested: CTAB 1 (modified from Saghai-Maroof et al., 1984), CTAB 2 (modified from Zoe Wilson, 2000), SDS1 and SDS 2 (both modified from Chao & Somers, 2012).

Composition of each isolation buffer is presented in Table 1. All isolation buffers had ultra pure water as solvent except CTAB 2 which had as solvent TRIS 0.2M.

Extraction protocols were performed as follows in Table 2.

Table 1. DNA extraction Buffer composition

Composition	CTAB1	CTAB2	SDS 1	SDS 2
TRIS	200 mM	-	1M	1M
NaCl	1400 mM	5%	-	
EDTA	20 mM	-	0.5M	5M
PVP	1.5%	-	-	1%
CTAB	2%	2%	-	
Sorbitol	-	2.5%	-	
Sarkosyl	-	1%	-	
EDTANa	-	0.8%	-	
SDS	-	-	2%	2%

Table 2. DNA isolation steps for used protocols
(*Optional step; **quantity according to pellet dimensions)

No	Step	CTAB 1	CTAB 2	SDS 1	SDS 2
1	Grounding seed	0.05g		0.05g	
2	Isolation buffer	500 µl		500 µl	
3	Incubation	60 min at 65°C		60 min at 65°C	
4	Cooling samples	5 min room temperature		5 min room temperature	
5	DNA purification	mix with 1 vol dichlormethan: isoamyl alcohol (24:1)		350 µl Potassium acetate 3M	
6	Centrifugation	15 min at 10000 RPM		10 min at 9000 RPM	
7	DNA purification	400 µl (vol:vol) dichlormethan: isoamyl alcohol (24:1)		400 µl (vol:vol) dichlormethan: isoamyl alcohol (24:1)	
8	Centrifugation	12 min at 10000 RPM		10 min at 9000 RPM	
9	DNA precipitation	5µl NaCl(5M)/100µl+2.5 vol EtOH kept at -20°C, samples on ice for 2-3 min (*)			
10	Centrifugation	6 min at 13000 RPM		6 min at 13000 RPM	
11	DNA pellet wash	200 µl Wash Buffer (76% EtOH, 10 mM NH_4OAC)		200 µl Wash Buffer (76% EtOH, 10 mM NH_4OAC)	
12	Centrifugation	5 min at 14000 RPM		5 min at 14000 RPM	
13	Pellet drying	30 min room temperature		30 min room temperature	
14	DNA solving	100 µl TE (**)		100 µl TE (**)	
15	DNA check	on agarose gel 0.8%			
16	RN-ase treatment	2 µl/100 µl probe		2 µl/100 µl probe	
17	Incubation	37°C for 1 h		37°C for 1 h	
18	DNA precipitation	2,5 vol EtOH kept at -20°C		2.5 vol EtOH kept at -20°C	
19	Centrifugation	5 min at 1400 RPM		5 min at 1400 RPM	
20	Pellet drying	30 min room temperature		30 min room temperature	
21	DNA solving	100 µl TE (**)		100 µl TE (**)	

Usually for DNA extraction protocols a mixture of chloroform:isoamyl alcohol is used for protein removal; in our protocols we substituted choloroform with dichlormethane.

Dichloromethane offers a viable alternative to chloroform in protocols for DNA extraction in which organic solvents are used; it has the same polarity index as chloroform but is less toxic and costs about half (Chaves et al., 1995).

Spectrophotometric measurements

DNA was quantified by measuring optical density at A260/A280 with spectrophotometer Beckman-Coulter Du Series 730.

After spectrophotometric measurements, probes (total DNA) were subjected to electrophoresis on 0.8% agarose gel and were photographed under UV light with BioPrint-Vilber Lourmant system.

PCR analysis

PCR analysis was performed in 20 µl of reaction mixture with MyTaq™ Red DNA Polymerase. A reaction tube contained 1.5 µl template DNA (25ng/ul), 0.6 U Taq polymerase, 4 µl reaction Buffer and 0.8 µl each primer. Amplifications were performed in the GeneAmp PCR System 9700 (Applied Biosystems) thermocycler with the following conditions:

- 95°C for 1 min; 35 cycles at 95°C for 15 sec, 50 °C for 15 sec, 72 °C for 10 sec and a final extension at 72 °C for 5 min for SSR marker AA175;
- 95°C for 2 min and 30 sec; 35 cycles at 95°C for 15 sec, 44 °C for 15 sec, 72 °C for 30 sec and a final extension at 72 °C for 5 min for ISSR marker 17899A.

Amplification products were separated in 2.5% agarose HR gel (1× Tris-Borate EDTA, stained with ethidium bromide) at a constant voltage of 80 V (90 min) for SSR marker AA175 and 1.5% agarose gel at a constant voltage of 100 V (60 min) for ISSR marker 17899A. The DNA bands were visualized and images were acquired using BioPrint-Vilber Lourmant system.

RESULTS AND DISCUSSION

Spectrophotometric results showed that DNA concentration were between 161-302 ng/µl for CTAB 1 protocol, 257-369 ng/µl for CTAB 2 protocol, 187- 432 ng/µl for SDS 1 protocol and 183-307 ng/µl for SDS 2 protocol.

Even if DNA concentration was highest in the case of CTAB 2 protocol, DNA quality was not as recommended by the standards, which require a ratio (A260/280) very close to 1.7.

By analyzing all data obtained with all extraction buffers, SDS1 is the most recommended for having good quality and quantity of total DNA.

Table 3. Spectrophotometric measurements

Sample	CTAB 1		CTAB 2		SDS 1		SDS 2	
	ratio	ng/µl	ratio	ng/µl	ratio	ng/µl	ratio	ng/µl
F95-928	1.360	161	1.071	369	1.791	432.17	1.662	187.16
Specter	1.168	278	0.996	360	1.735	286.39	1.783	307.50
Windham	1.303	302	0.893	321	1.718	214.75	1.685	183.36
Nicoleta	1.096	222	0.995	257	1.813	187.29	1.732	227.22
Average	**1.231**	**241**	**0.988**	**327**	**1.764**	**280.15**	**1.715**	**226,31**

Comparison between spectrophotometric readings and electrophoresis profiles did not show similar results, particularly in case of CTAB 1 and CTAB 2 protocols, in which electrophoretic profiles showed a small DNA quantity (Figure 1).

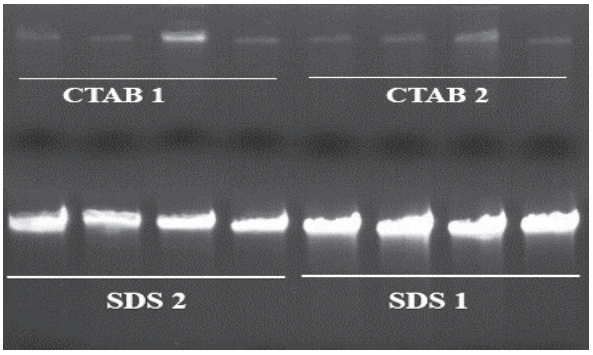

Figure 1. Electrophoresis of total DNA extracted from Pea seed using CTAB1, CTAB2, SDS1 and SDS2 method in 0.8% agarose gel

PCR amplification

In order to determine whether isolated DNA could be used for molecular markers analysis, PCR amplification with two types of primers was performed (marker AA 175 and 17899A).

First PCR analysis was conducted with ISSR marker 17899A. The results showed good amplification for samples isolated using SDS 1 and SDS 2 protocol.

Samples isolated using protocol CTAB 1 and CTAB 2 din not show amplification products (Figure 2).

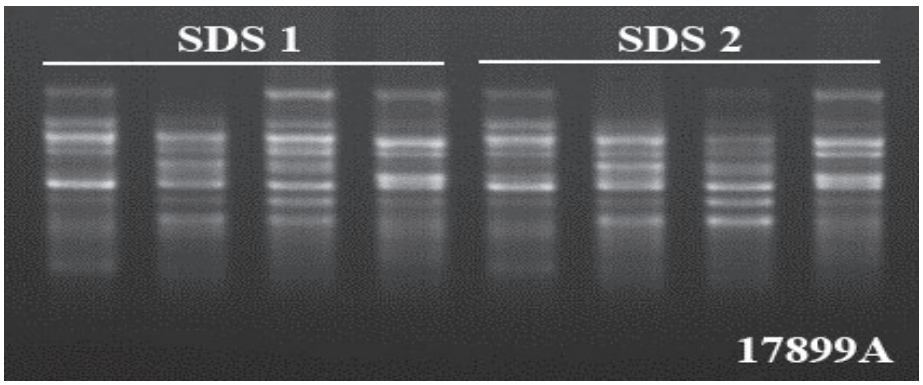

Figure 2. Amplified band patterns obtained with ISSR marker 17899A (F95-927, Specter, Windham and Nicoleta)

PCR with specific marker AA 175 (Loridon K, 2005) also showed good amplification for samples isolated using SDS extraction methods and did not show amplification products for samples isolated with CTAB extraction methods (Figure 3).

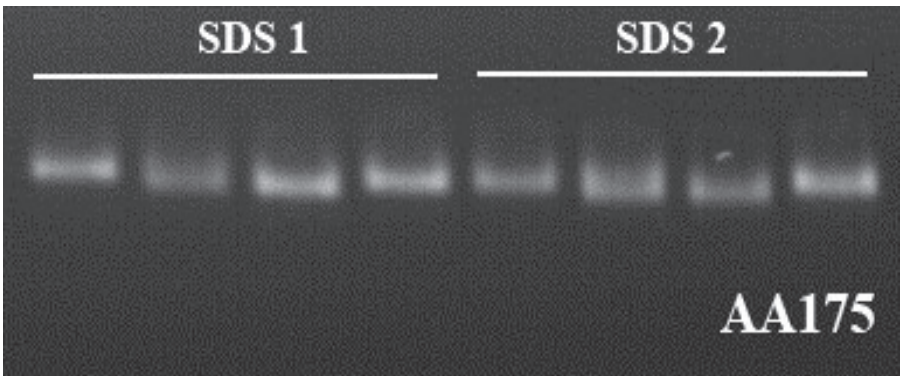

Figure 3. Amplified band patterns obtained with SSR marker AA175 (F95-927, Specter, Windham and Nicoleta).

CONCLUSIONS

None of the two extraction methods based on CTAB buffer was suitable for extracting good quality DNA from dry pea seeds.

By analysing the results obtained in this study, SDS1 proved to be the most suitable method for obtaining pure and intact DNA (from dry pea seeds), which can be used for PCR analysis. More than that, SDS 1 method is less expensive, since the use of PVP brings no improvement to the method SDS2.

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