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Random amplified polymorphic DNA technique for detection of plant based adulterants in chilli powder (*Capsicum annuum*).

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Abstract

The present study reports a modified protocol for isolation of good quality DNA from chilli powder and its probable adulterants (dried red beet pulp, almond shell dust and powdered *Ziziphus nummularia* fruits) and the utility of RAPD primers for detection of adulterants in marketed chilli powder. Selected RAPD primers, which produced adulterant specific bands in simulated samples were used for analyzing market samples of chilli powder. Out of the six market samples analyzed, one sample amplified *Z. nummularia* specific band indicating the occurrence of adulteration in market samples. All the market samples tested were free from dried red beet pulp or almond dust adulteration.

Key words: adulterant detection, chilli powder, DNA isolation, PCR, RAPD

Introduction

Chilli (*Capsicum annuum*) is an indispensable culinary spice used all over the world and is valued principally for its pungency and colour. It is also used in beverages and in medicinal preparations (Berke & Shieh, 2001). India is one of the world's largest producer, consumer and exporter of chillies. Chilli is exported as dry whole fruits, crushed chilli, chilli powder and its value added products like fermented chilli, chilli paste, oleoresin etc. It is estimated that around 20-30 percent of chilli crop in India is used for powder preparation. India exports around 22000 tons of chilli powder per year (Spices Statistics, 2004).

Various types of adulteration are possible in chilli and have been reported at various times. The chilli powder and paste is more vulnerable to adulteration as foreign substances can go into it visually undetected (Chakrabarti & Roy, 2003). Chilli powder is reported to be adulterated by adding extra amounts of bleached pericarp, seeds, calyx and peduncle to increase bulk without visually affecting the appearance (Govindarajan, 1986). Other reported adulterants include artificial dyes (coal tar red and sudan red), almond shell dust, red beet pulp (Berke & Shieh, 2001) and dried and powdered fruits of *Ziziphus nummularia* (personal communication). Even though

spectrophotomertic and HPLC methods are available for the detection of artificial dyes in chilli powder, at present no technique is available to detect the plant based extraneous materials in chilli powder.

With the advance of biotechnology, molecular tools are now considered to provide more precise, quick and reliable analytical method for adulterant detection and authentication of food items. [Bryan *et al.* (1998); Heather (2000); Lockley & Bardley (2000); Clavo et al. (2001); Terzi *et al*. (2003); Bandana and Mahipal (2003); Sasikumar *et al*. (2004); Chang-Chai *et a*l. (2005); Aida *et al.* (2007); Dhanya *et al.* (2007)]. RAPD fingerprints have been successfully used for identification of adulterants in processed spices (Sasikumar *et al*. 2004) and poultry pates (Clavo *et al.* 2001).

Good quality amplifiable DNA is a prerequisite for molecular based technique. Since chilli powder and their possible adulterants like dried red beet pulp, almond shell dust and dried *Z. nummularia* are recalcitrant materials, isolation of good quality DNA from these materials needs to be standardized. Good quality DNA isolation from recalcitrant tissues is hindered by the presence of polysaccharides, proteins, polyphenols, alkaloids and other secondary metabolites. The dry nature of the sample also increases the problems in DNA isolation. In the present study we report a modified CTAB protocol for isolating good quality DNA from chilli powder including market samples, probable adulterants (dried red beet pulp, almond shell dust and dried *Z. nummularia*) and simulated samples and development of a PCR based method for detection of adulteration in chilli powder. This work is relevant as adulteration is a major concern in the context of the sanitary and phytosanitary issues of WTO agreement and the importing countries are imposing strict quality standards for produces.

Material and methods

Sample materials

Dried chilli fruits (*Capsicum annuum*) obtained from the local market is used as genuine sample in the study. Almond shell dust was prepared from Indian almond fruits (*Terminalia catappa*) obtained from IISR experimental farm, Peruvannamuzhi. Dried red beet pulp was made from beet root (Beta vulgaris) obtained from the market. Fruits of *Z. nummularia* used in the study were obtained from a reliable source. All the samples were ground to fine powder using Cyclotech 1093 sample mill. Three simulated samples of chilli powder A (chilli: red beet pulp), B (chilli: almond shell dust) and C (Chilli: *Z. nummularia*) in the proportion 95:5 prepared on a weight basis were used for adulterant detection study. The six market samples of chilli powder were also obtained from market.

DNA isolation

DNA was isolated from all the above samples using the CTAB protocol (Remya *et al*., 2004) with minor modifications.

1) Weigh 2 grams of powdered sample in 50 ml oakridge tube.
2) Mix the powder with 10 ml of alcohol (95%) for 5 minutes at room temperature.
3) Centrifuge at 5000g for 5 minutes.
4) Decant the supernatant and repeat the washing step.
5) Dry the powder thoroughly in an air flow and homogenize the sample in 10 ml of extraction buffer (3% CTAB, 100 mM Tris-HCl (pH 8), 20 mM EDTA (pH 8), 2 M NaCl and 0.3 % ß- mercaptoethanol)
6) Incubate at 65oC in a water bath for 1 h with intermittent shaking.
7) Add 10 ml of phenol: chloroform: isoamyl alcohol (25:24:1) and mix by inversion for 10 min.
8) Centrifuge at 8,000 g for 15 min at 4°C.
9) Transfer the aqueous phase to a fresh Oakridge tube and add equal volume of chloroform: isoamyl alcohol (24: 1) and mix by inversion for 10 min.

10) Centrifuge at 8,000 g for 15 min at 4°C.

11) Transfer the aqueous phase to a fresh Oakridge tube and add equal volume of ice cold ethanol. Incubate at - 20°C for 2 hr to precipitate the DNA.

12) Pellet down the DNA at 5,000 g for 5 min and wash the pellet with 70 per cent ethanol.

13) Dry the pellet and dissolve in minimum volume of sterile double distilled water and add 10 µg/ml of RNase and incubate at 37°C for 1 h.

14) Add equal volumes of phenol: chloroform: isoamyl alchohol (25:24:1). Mix for 15 min. Centrifuge at 10,000 g for 15 min at 4°C.

15) Transfer the aqueous phase and repeat the extraction once again.

16) Take the aqueous phase and add equal volume of chloroform: isoamylalcohol (24: 1) and mix well.

17) Centrifuge at 10,000 g for 15 min at 4°C

18) Take out the aqueous phase and add equal volumes of 100 per cent ice-cold ethanol and leave at - 20°C for 2 h to precipitate the DNA.

19) Pellet down the DNA at 10,000 g for 5 min and wash with 70 per cent ethanol to remove salts.

20) Dry and dissolve the DNA pellet in 500 µl of DNase free water.

21) Add 200 µl 30 per cent PEG 8000 to DNA sample and keep in room temperature for 30 min.

22) Centrifuge at 14,000 g for 15 min.

23) Wash the pellet with 80 per cent ethanol, dry off the ethanol and dissolve the pellet in nuclease free water or Tris-EDTA (10: 1) buffer.

24) Store the samples frozen at - 20°C.

The concentration and quality of the DNA isolated from all the samples were examined by agarose gel electrophoresis. The quality of the DNA was also checked in spectrophotometer by taking the absorbance ratio at 260/280 nm UV.

RAPD analysis

Amplification of the DNA isolated from all the sample tissues was done by using random decamer primers obtained from OPERON Technologies, Alameda, USA. The RAPD reaction was performed in 25 µl reaction volume with 35 ng genomic DNA, 0.2 mM dNTPs, 0.3 µM primer, 2 mM $MgCl_2$, 1X Assay buffer and 1 U Taq DNA polymerase using a PTC - 100 programmable thermal controller, M J Research, Inc, USA, according to Willams *et al.* (1990). The amplified products were visualized by electrophoresis in a 2% agarose gel containing 0.5 µg/ml of ethidium bromide and documented by a gel documentation system (Alpha Imager 2220, USA).

Screening and selection RAPD primer

Fifteen random decamer primers were screened for amplification in genuine chilli and probable adulterants (dried red beet pulp, almond shell dust and *Z. nummularia*). The primers which gave consistent amplification pattern for both chilli and adulterants separately were selected for subsequent amplification. Genomic DNA from each simulated sample along with genuine chilli powder and respective adulterant were amplified and resolved in the same agarose gel to identify the adulterant specific bands. Primers which produced adulterant specific bands in simulated samples of chilli powder were used for screening six market samples of chilli powder under the same reaction condition.

Results and discussion

Isolation of good quality DNA from any recalcitrant tissue is a difficult task and can be achieved only by trial and error methods. Though we have tried different DNA isolation protocols (Singh *et al.*, 1999; Cheng *et al.*, 1997; Schneerman *et al.*, 2002; Sangwan *et al.*, 2000; Pirtilla *et al.*, 2001; Doyle and Doyle, 1987, Remya *et al.*, 2004) for chilli powder and

possible adulterants none of them except Remya *et al*. (2004) yielded DNA from all the sample tissues. Hence this protocol of Remya *et al*. (2004) was slightly modified to yield sufficient amount of good quality DNA. The initial ethanol wash (Steps 2, 3 and 4) in our protocol could remove the colouring pigments in the samples tissues (except betalin pigments in the dried beet pulp) to a great extent. In many plants it is observed that increased CTAB and NaCl concentrations increase the yield of cellular DNA (Smith *et al*., 1991). Our study also supports this statement. The additional steps of phenol: chloroform: isoamyl extractions (step 7) and chloroform: isoamyl extractions (steps 9 and 16) in our protocol improved the quality of DNA as chilli samples are rich in protein content. The precipitation of DNA with ethanol instead of isopropanol in steps 11 and 18 increased the yield of DNA. The faint colour of the DNA preparation from the beet root pulp can be removed by adding a pinch of activated charcoal to the DNA preparation followed by centrifugation (this step is optional as this does not affect the amplification of DNA).

The modified CTAB protocol could yield sufficient quantity genomic DNA from dried chilli powder including the genuine chilli powder, market samples and simulated samples, dried red beet pulp, almond shell dust and powder of dried fruits of *Z. nummularia*. A high A260/280 ratio observed in all the samples indicated the purity of the DNA. Table 1 show the yield and quality of DNA isolated using the modified protocol. Agarose gel electrophoresis of DNA samples showed conspicuous bands of high molecular weight DNA with a little shearing.

Out of fifteen RAPD primers screened for amplification in genuine chilli and probable adulterants, eleven primers (OPA-02, OPA-08, OPA-10, OPA-12, OPA-13, OPA-15, OPC-07, OPC-08, OPD-05, OPD-11and OPJ-18) which gave good amplification in all the samples were further used for amplifying simulated samples of chilli powder (samples A, B and C). Amplified products were scored on the basis of major bands present. Bands specific to adulterant were scored separately on the basis of their presence in the adulterant and simulated sample and absence in the genuine chilli powder. Among the eleven primers used, four primers (OPA-02, OPA-15, OPC-08 and OPJ-18) produced red beet pulp specific bands in simulated sample A, two primers (OPA-02 and OPC-07) produced almond dust specific bands in simulated sample B and three primers (OPA-10.OPA-12 and OPC-07) produced *Z. nummularia* specific bands in simulated sample C. Primers OPA-08, OPA-13, OPD-05 and OPD-11 did not produce adulterant specific bands in any of the simulated samples. The sequence of RAPD primers and size of adulterant specific fragments generated by different RAPD primers in simulated samples are given in Table 2.

Table 1. Yield and quality of DNA isolated from different samples using the modified protocol.

Sample	Yield of DNAµg/g dry powder	A260/280
Genuine chilli	17	1.85
Red beet pulp	6	1. 67
Almond shell dust	8	1. 82
Ziziphus nummularia	10	1.74
Simulated sample A	14	1.88
Simulated sample B	11	1.75
Simulated sample C	13	1.73
Market sample1	15	1.9
Market sample 2	16	1.83
Market sample 3	12	1.71
Market sample 4	14	1.85
Market sample 5	15	1.69
Market sample 6	13	1.75

Among the adulterant specific bands obtained by different primers in three simulated samples, specific bands produced by primer OPA-02 [red beet pulp (~400bp and -850bp) (Fig. 1) and almond shell dust (~450bp)], OPA-15 [red beet pulp (~410bp)], and OPA10 [Ziziphus nummularia (~420bp)] were found

Table 2. The sequence of RAPD primers and size of adulterant specific fragment produced in simulated samples.

Primer	Sequence	Adulterant specific bands (~ Size in bp)		
		Simulated sample A (Chilli :Dried beet pulp)	Simulated sample B (Chilli :Almond shell dust)	Simulated sample C (Chilli : Z. nummularia)
OPA-02	5'TGCCGAGCTG 3'	400	450	-
OPA-08	5'GTGACGTAGG3'	-	-	-
OPA-10	5'GTGATCGCAG 3'	-	-	420
OPA-12	5'TCGGCGATAG3'	-	-	440
OPA-13	5'CAGCACCCAC3'	-	-	-
OPA-15	5'TTCCGAACCC 3'	850, 410	-	-
OPC-07	5'GTCCCGACGA 3'	-	400	390
OPC-08	5'TGGACCGGTG3'	820	-	-
OPD-05	5'TGAGCGGACA3'	-	-	-
OPD-11	5'AGCGCCATTG3'	-	-	-
OPJ-18	5'TGGTCGCAGA3'	315	-	-

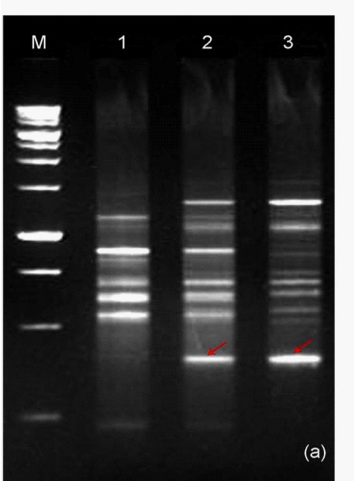

Fig. 1. RAPD profiles of genuine chilli, simulated samples and probable adulterants using different primers (a) RAPD profile of Genuine chilli, simulated sample A (chilli: red beet pulp) and red beet pulp with primer OPA-02. Lane1- Genuine chilli, Lane2- Simulated sample A, Lane 3 - Red beet pulp, M - 1 Kb DNA ladder (Biogene, USA).

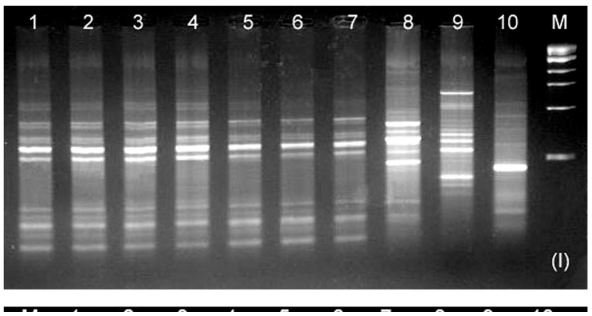

Fig. 2. RAPD Profile of genuine chilli, market samples and probable adulterants with primers OPA-02(I) Lane 1- Genuine chilli, Lane 2- Market sample 1, Lane 3- Market sample 2, Lane 4- Market sample 3, Lane 5- Market sample 4, Lane 6- Market sample 5, Lane 7- Market sample 6, Lane 8 - Z. nummularia, Lane 9 - Dried beet pulp, Lane 10 - Almond shell dust. M- 1 Kb DNA ladder (New England Biolabs).

unique and easily distinguishable. These three primers were used for analyzing six market samples of chilli powder for the presence of adulterants (dried beet pulp/ almond shell dust /*Z. nummularia*). Interestingly, one of the market samples (Sample no.5) produced a *Z. nummularia* specific band (~420bp) with RAPD primer OPA10 indicating the presence of adulterant in the sample. Red beet pulp and almond shell dust specific fragments were absent in all the six market samples studied.

The extraction of DNA from food matrices for subsequent use in PCR is often considered to be a problem. Our work has shown that DNA extracted from chilli powder, by the

method described, can be successfully used as template DNA in PCR. The PCR method using random primers could detect the presence of *Z. nummularia* in one of the market samples of chilli powder confirming the occurrence of adulteration in commercial samples of chilli powder with *Z. nummularia* fruits. But all the six samples tested were free from dried beet pulp and almond shell dust adulteration.

The result showed that the RAPD-PCR is a good method for identification of plant based adulterant in chilli powder and could detect adulteration at a concentration level of more than 50g/Kg of chilli powder. However, further research is needed to study the detection limit. The adulterant specific bands identified in the present study can be cloned and sequenced to produce specific Sequence Characterized Amplified Region (SCAR) markers which could be used for large scale screening of powdered chilli samples and this can be commercialized as an analytical method to detect the purity of the exported consignments of chilli powder besides its use in produces for the domestic market.

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