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Full Length Research Paper

Polyphenolics free DNA isolation and optimization of PCR-RAPD for fennel (*Foeniculum vulgare* Mill.) from mature and young leaves

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Molecular analysis of fennel (*Foeniculum vulgare* Mill.) strictly relies on high yield, and good quality high molecular weight DNA samples. DNA was isolated from the mature and fresh young tender leaves obtained from various Italian wild populations of fennel. We performed a modified cetyl trimethyl ammonium bromide (CTAB) protocol introducing several modifications such as inclusion of variable percentage of polyvinylpyrrolidone (PVP, 2 - 6%), different molarity of sodium chloride (NaCl, 1.5 - 4 M), activated charcoal (1 to 2%), and pre-heated buffer (65°C) with and without liquid N2 extraction for the mature leaves. In contrast, the CTAB protocol without any additional anti-oxidants using liquid N_2 extraction was performed for the fresh young leaf tissue. Optimization of polymerase chain reaction-random amplification of polymorphic DNA (PCR-RAPD) conditions included 10X PCR buffer compositions such as $(NH_4)_2SO_4$ with 0.1% (v/v) Tween 20 over KCl buffer with and without 0.8% (v/v) Nonidet P40 and an 'optimized' buffer which contains KCl and $(NH_4)_2SO_4$, $MgCl_2$ (2.5 mM), Taq enzyme (1 to 1.5 U), annealing temperature of 37 and 42°C and PCR reaction volume of 10 and 25 µl. The results show that DNA isolated from fresh young leaves were superior in quality and quantity over mature (stored) leaves and was amenable to optimized PCR-RAPD conditions.

Key words: Activated charcoal, cetyl trimethyl ammonium bromide (CTAB)-DNA, *Foeniculum vulgare*, NaCl, polymerase chain reaction-random amplification of polymorphic DNA (PCR-RAPD), PCR buffer, polyphenolics, polyvinylpyrrolidone (PVP).

INTRODUCTION

Fennel (*Foeniculum vulgare* Mill.) is an aromatic plant belonging to the family Apiaceae which is widespread in the whole Mediterranean basin. This plant is well known for its essential oil and high polyphenolic content (Oktay et al., 2003; Parejo et al., 2004; Krizman et al., 2006a). Fennel is used to flavor liquors, bread, fish, salad, soups, cheese, meat and is used in the manufacturing of pickles, perfumes, soaps, cosmetics and cough drops (Tanira et al., 1996; Beaux et al., 1997; Garcia-Jimenez et al., 2000; Patra et al., 2002).

Many of the medicinal and aromatic plant species are undergoing domestication and cultivar development. Therefore, it is desirable to use molecular markers for the genetic improvement programs by choosing parents and selecting progeny. To achieve this goal, a good quality and quantity DNA free of any interfering contaminants is required. The high content of polyphenols, polysaccharides, tannins, sugars and carragenans and other secondary metabolites from aromatic plants can hamper the DNA isolation procedures and thus affect the subsequent downstream applications especially for PCR based techniques (RAPD). Some of the problems encountered during the isolation and purification of high molecular weight DNA from mature leaf tissue include

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Abbreviations: CTAB, Cetyl trimethyl ammonium bromide; **PVP,** polyvinylpyrrolidone; **PCR-RAPD,** polymerase chain reaction-random amplification of polymorphic DNA.

degradation of DNA due to endonucleases, co-isolation of highly viscous polysaccharides and co-precipitation of various inhibitor compounds such as polyphenols and other secondary metabolites which directly or indirectly can interfere with the subsequent enzymatic reactions (Weising et al., 1995). In addition, the contaminating RNA that eventually precipitates along with DNA may cause many problems including suppression of PCR amplification (Pikkart and Villeponteau, 1993) and interference with RAPD analysis (Mejjad et al., 1994). Previous study have shown that DNA extracts from tissue past the budding stage (mature) are problematic with the PCR amplification and are less stable under long-term storage (Lodhi et al., 1994). The presence of polyphenols, which are powerful oxidizing agents present in many plant species, can greatly reduce the yield and purity of extracted DNA (Loomis, 1974; Porebski et al., 1997). These contaminants can co-precipitate with DNA in the extraction procedure and thereby inhibit DNA digestion and PCR (Zhang and McStewart, 2000), presumably by irreversible binding with DNA (Dabo et al., 1993). Until now, various protocols for DNA extraction have been successfully applied to many plant species (Doyle and Doyle, 1987; Ziegenhagen and Scholz, 1993), which were further modified to provide DNA suitable for several kinds of analyses (Wang and Taylor, 1993; Ziegenhagen and Scholz, 1998). Plant species belonging to the same or related genera can exhibit enormous variability and complexity in the biochemical pathways of dispensable functions. The chemotypic heterogeneity among species may not permit optimal DNA yield with unique isolation protocol. Thus, even closely related species of the same genus may require different isolation protocols (Weising et al., 1995). The PCR based method for DNA profiling techniques viz., RAPD (Welsh and McClelland, 1990; Williams et al., 1990; Mir Ali and Nabulus, 2003; Fracaro et al., 2005) have been extensively applied in the genetic diversity analysis with several modifications in various plant species.

Most of the protocols suggest extraction of DNA from fresh young leaf materials owing to the fact that young leaves provide better quality and yields of DNA than older leaves. Though sometimes, rare samples collected from different remote locations and/or unavailability (seasonal dependence) of young leaf materials force us to work on mature leaves which consist of plant parts in dry/semi-dry condition (during transportation). In the present study, we evaluated, optimized cetyl trimethyl ammonium bromide (CTAB)-DNA isolation and polymerase chain reaction-random amplification of polymorphic DNA (PCR-RAPD) protocols introducing several modifications to the original protocol (Doyle and Doyle, 1987; Murray and Thompson, 1980; Williams et al., 1990).

These modifications included addition of polyvinylpyrrolidone (PVP), NaCl, and activated charcoal for the mature leaves while for the fresh young leaf tissue, a simple CTAB protocol without any additional anti-oxidants (PVP) was performed. Subsequently, PCR-RAPD protocols have been carefully modified and optimized with respect to PCR ingredients such as PCR buffer, $MgCl_2$, *Taq* enzyme, 10-mer primer and temperature annealing, PCR reaction volume as well PCR cycling conditions for the successful amplification of DNA.

MATERIALS AND METHODS

Plant sampling

Fennel mature leaves and fresh young tender leaves were collected from the wild in various places (Visso, Camerino, Matelica and San Severino) of the province of Macerata, Italy. The mature leaves were collected during winter season (October to November), and transferred to the laboratory of Molecular Ecology, University of Camerino, Camerino, Italy for the biochemical analysis. Collected mature leaves were immediately processed for the DNA isolation or stored properly at -20°C in a zip lock polythene bag for future use. The fresh young leaves were collected during spring season (May to June) in a zip lock polythene bag, transferred to the Molecular Ecology laboratory, University of Camerino, Camerino, Italy and were immediately processed for the DNA extraction or stored at -20°C for further analysis.

Optimization of DNA isolation protocols

Genomic DNA from the fresh young tender leaves of each sample was extracted with CTAB, as described earlier (Doyle and Doyle, 1987; Murray and Thompson, 1980) with minor modifications. The fresh young leaf tissue (200 to 300 mg up to 2 g) was frozen with liquid N_2, ground to a fine powder, mixed with preheated (65°C) extraction buffer [2% (w/v) CTAB, 20 mM ethylene diamine tetra-acetic acid (EDTA), 1.4 M NaCl, 100 mM Tris, pH 8.0 containing 3% β-mercaptoethanol], at 1:4 or 1:5 sample buffer ratio, and shaken gently at 65°C for 15 to 30 min. An equal volume of 24:1 (v/v) chloroform: isoamyl alcohol was added and mixed gently for 15 min then centrifuged at 7,000 x *g* for 20 min at room temperature (24°C). The aqueous phase was transferred to a new tube and an equal volume of ice-cold isopropanol was added and mixed gently till the clump of DNA fibrils were obtained. The DNA was spooled out or precipitated by centrifugation and then washed with 80% (v/v) ethanol; air-dried and finally dissolved in 100 to 500 µl 1X TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). To remove contaminating RNA, 5 µl of RNase A (10 mg/ml) was added and incubated overnight at 37°C. The RNase digested DNA was extracted with equal volumes of phenol : chloroform : isoamylalcohol (25:24:1, v/v/v) at 12,000 rpm for 5 min. The aqueous layer was transferred to a fresh tube and re-extracted with equal volume of chloroform : isoamyalcohol (24:1) or chloroform by centrifuging at 12,000 rpm for 5 min. Then, the supernatant was transferred to a fresh tube, equal volumes of absolute alcohol was added and then 0.1 volume of sodium acetate, at pH 5.3 and was incubated at -80°C for 1 h followed by centrifugation at 12,000 rpm for 10 min. This was then followed by 80% ethanol (twice) wash to remove residual salt. The pellet was air dried and re-suspended in 0.1X TE buffer (1 mM Tris, 0.1 mM EDTA, pH 8.0).

The following modifications were attempted in the CTAB-DNA extraction buffer for the mature leaves with the above-described methodology. They are; 1) inclusion of anti-oxidant from 2 to 6% (+1% increment) polyvinylpyrrolidone (PVP, 360,000 average molecular weight, Sigma-Aldrich, Missouri, USA) (Dellaporta et al., 1983; Weising et al., 1995; Kim et al., 1997; Puchooa and Venkatasamy, 2005; Shinde et al., 2007), 2) gradual increase in the

concentration of β-mercaptoethanol between 2 to 5% (+1% increment) (Puchooa, 2004), and 3) increasing salt concentration of sodium chloride (NaCl) from 2 to 4 M (+0.5 M increment) (Fang et al., 1992; Harini et al., 2008; Krizman et al., 2006b). In addition, inclusion of 1 to 2% activated charcoal RBW II (FeinBiochemica, Heidelberg, New York, USA), preheated buffer (55 to 65°C), and grinding alternative to liquid nitrogen along with a 'wash solution' step (15 mM ammonium acetate in 75% (v/v) ethanol) was performed (Krizman et al., 2006b).

DNA quantification and dilutions

The purified DNA samples were measured at OD A_{260} and A_{280} using Genesys 10 UV spectrophotometer (Thermo Electron Corporation, Madison, USA). The purity of the DNA was assessed by calculating the ratio of OD A_{260}/A_{280}. DNA concentration and integrity was also determined by running the samples on 0.8% agarose gel and the concentration was evaluated based on the intensities of band with reference to the molecular weight standard Lambda DNA EcoRI HindIII digest (MBI, Fermentas Inc, Maryland, USA) or 1 kb gene ruler (USB Corporation, Ohio, USA). The nucleic acid concentration was calculated following Sambrook et al. (1989) and the DNA samples were diluted to 10 or 20 ng/µl for PCR amplification.

Optimization of PCR-RAPD parameters and amplification conditions

The protocol described previously (Williams et al., 1990) was followed for PCR-RAPD amplification with necessary modifications and are briefly described in Table 1. Oligonucleotide sequences (10-mers) were obtained from Operon Technologies Inc. (Alamada, CA, USA) and were synthesized by Sigma Genosys, Sigma-Aldrich, Missouri, USA. Apart from the routinely employed PCR-RAPD parameters such as magnesium chloride ($MgCl_2$), *Taq* enzyme, deoxynucleotide triphosphates (dNTPs, fixed), primer (fixed), template DNA, final PCR reaction volume and other PCR cycling conditions (Table 1), here we attempted, for the first time, optimization of different composition of 10X PCR buffer which included a) 750 mM Tris-HCl (pH 8.8 at 25°C), 200 mM $(NH_4)_2SO_4$, 0.1% (v/v) Tween 20 (MBI, Fermentas Inc, Maryland, USA), b) 100 mM Tris-HCl (pH 8.8 at 25°C), 500 mM KCl, 0.8% (v/v) Nonidet P40 (MBI, Fermentas Inc, Maryland, USA), c) optimized dream Taq buffer containing KCl and $(NH_4)_2SO_4$ with 20 mM $MgCl_2$ (MBI, Fermentas Inc, Maryland, USA) and d) 100 mM Tris-HCl (pH 8.6), 500 mM KCl, 15 mM $MgCl_2$ (USB Corporation, Ohio, USA).

The optimum PCR-RAPD amplification reaction was performed in a 25 µl volume containing 40 to 50 ng genomic DNA, 200 µM dNTP mix (MBI, Fermentas Inc, Maryland, USA), 15 pm single 10-mer primer, 1X PCR buffer [10X containing 750 mM Tris-HCl (pH 8.8 at 25°C), 200 mM $(NH_4)_2SO_4$, 0.1% (v/v) Tween 20, MBI, Fermentas Inc, Maryland, USA)], 2.5 mM $MgCl_2$ (25 mM, MBI, Fermentas Inc, Maryland, USA) and 1 to 1.5 unit of Taq DNA polymerase (5 U/µl, MBI, Fermentas Inc, Maryland, USA and USB Corporation, Ohio, USA). PCR-RAPD amplification was performed in a MyCycler™ Thermal Cycler (Bio-Rad Laboratories Inc., Hercules, California, USA) and TGRADIENT Thermocycler (Biometra, Göttingen, Germany) with an initial denaturation at 95 or 94°C for 3 min, followed by 35 to 40 cycles of 94°C for 1 min, 37 and 42°C for 1 min, and 72°C for 1 min 10 s, with a final extension at 72°C for 15 min, and lastly a hold temperature at 4°C. PCR-RAPD reactions were repeated thrice in order to ensure reproducibility.

Gel electrophoresis

The PCR-RAPD amplified products were subjected to 1.5% agarose gel electrophoresis using 1X Tris-acetate-EDTA (TAE) buffer (40 mM Tris-acetate, pH 8.0, 1mM EDTA) at 75 V for 3 to 5 hr. The gels were stained with 0.5 mg/ml ethidium bromide, and visualized on ultraviolet 2011 Macrovue Transluminator (LKB, Bromma, Sweden). The banding patterns were captured and analyzed using Kodak Digital Science DC40, 1D software for Electrophoresis Documentation and Analysis System (Kodak, Rochester, New York, USA).

Gel extraction

In the case of phenolic carryover, DNA was gel extracted by conventional, simple freeze-squeeze method and purified, precipitated with 0.1 volume of 3 M sodium acetate (pH 5.3) and 2.5 volumes of ice-cold ethanol, followed by 80% ethanol to remove residual salt. The final pellet was air dried and re-suspended appropriately.

RESULTS AND DISCUSSION

DNA isolation from mature leaves and constraints

The genomic DNA extracted from the mature leaves by CTAB method without any modification gave good DNA yield as observed on 0.8% agarose gel electrophoresis (Figures 1g to i). However, the spectrophotometric measurement at OD A_{260}/A_{280} that ranged between 1.2 to 1.5 revealed the poor quality of DNA with which PCR-RAPD failed to amplify (Figure 1j). Hence, it was not applicable to the downstream applications. In fact, the presence of polyphenols, which are powerful oxidizing agents present in many plant species, can reduce the yield and purity by binding covalently to the extracted DNA making it useless for most research applications (Katterman and Shattuck, 1983; Peterson et al., 1997; Porebski et al., 1997). The failure of PCR-RAPD amplification is probably due to the phenolic carryover (browning effect) (Figures 1b to e), presumably by irreversible interactions with DNA (Dabo et al., 1993). Upon electrophoresis of the contaminated DNA, a co-migrating yellow coloration (Figure 1k) was observed confirming the presence of polyphenolics, which could be attributed to the change in the pH (acidic) (Sambrook et al., 1989). Henceforth, it is required for a more efficient DNA isolation and PCR-RAPD protocol for molecular characterization especially from the mature leaves. Further, the phenolic DNA was gel extracted and purified by simple freeze-squeeze method (Figures 1l and m). This was then followed by a PCR-RAPD which gave amplification exclusively at low molecular weight base pairs (Figure 1n). At this stage, it was confirmed that the phenolic carryover (viz., yellowish-brown colouring) led to the inhibition of PCR-RAPD. In addition, we observed that most of the genetic information was lost due to the aforementioned procedure. Hence, subsequent attempts with several modifications were made with each parameter tested at a time in order to remove the phenolic carryover as well as other possible contaminating substances. Inclusion of antioxidants in the DNA

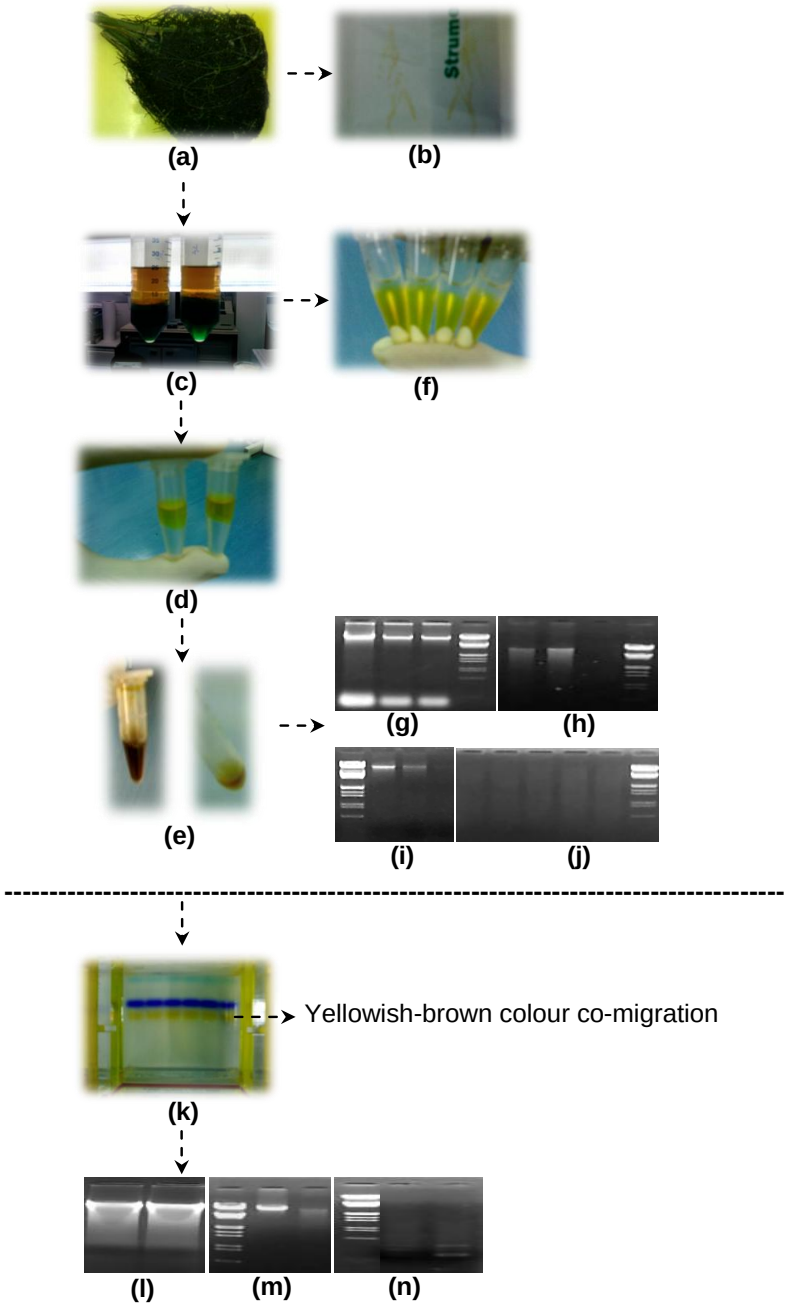

Figure 1. **DNA** isolated from the mature leaves and subsequent encountered problems. (a) Mature leaves; (b) phenolics released on the weighing paper; (c) supernatant with strong phenolic carryover (browning), after chloroform: isoamyl alcohol (24:1) extraction; (d) after chloroform extraction; (e) RNase A digested, purified DNA with phenolic carryover; (f) high salt precipitation (4 M NaCl) method; (g) undigested DNA showing RNA contamination; (h and i) RNase A digested DNA without any RNA (activated charcoal method and CTAB-PVP method, respectively); (j) subsequent PCR-RAPD analysis inhibited by phenolic carryover; (k) gel extraction of phenolic-DNA, the yellow colour co-migration depicts change in pH (acidic) due to phenolics; (l) gel picture showing concentrated DNA with smear; (m) gel extracted, purified DNA and its concentration; (n) PCR-RAPD of the purified DNA, showing low molecular weight amplicons (Note: Loss of high molecular weight information). CTAB, Cetyl trimethyl ammonium bromide; PVP, polyvinylpyrrolidone; PCR-RAPD, polymerase chain reaction-random amplification of polymorphic DNA.

extraction buffer such as β-mercaptoethanol, ascorbic acid, bovine serum albumin (BSA), sodium azide and PVP are commonly used to deal with problems related to phenolics (Dawson and Magee, 1995; Clark, 1997). We adopted a high salt concentration (2 to 4 M NaCl) to prevent or to diminish the dissolution of polysaccharides during the extraction step (Fang et al., 1992; Harini et al., 2008; Krizman et al., 2006b). In an attempt to promote high-molecular weight DNA isolation free of interfering contaminants, we introduced few other modifications related to; 1) sample buffer ratio, 2) use of β-mercapto-ethanol (2 to 5%), 3) inclusion of polyvinylpyrrolidone (PVP, Mr 360,000, 2 to 6%) or activated charcoal or combination of both (Martellossi et al., 2005; Krizman et al., 2006b). Furthermore, we also focused to keep the protocol as simple as possible, to minimize the number of steps involved.

Technical challenges and improvements

Following the aforementioned modifications, it was obvious that there were no improvement in the overall quality of DNA viz., PCR usable (Figures 1g to j) obtained from mature leaves indicating that the developmental stage of the plant should be considered strictly before proceeding fennel molecular characterization work which are known for its secondary metabolites. Interestingly, we obtained a good quality that is, PCR amplifiable DNA (Figure 1h) from the mature leaf tissue while working with 2% PVP along with activated charcoal. The result obtain is in agreement with Krizman et al. (2006b). The incur-poration of activated charcoal in the extraction mixture before sample incubation greatly increased the chance for DNA to be PCR amplifiable (Bi et al., 1996), most likely by preventing irreversible interaction of DNA with polyphenolic substances, since cytosol-borne compounds might come in contact with activated charcoal earlier than DNA. It was generally assumed that PVP in the presence of activated charcoal has a synergistic effect to bind polyphenolics (Krizman et al., 2006b). As it has been already used as a polyphenolics-binding agent (Maliyakal, 1992; Bi et al., 1996; Porebski et al., 1997; Peterson et al., 1997; Kim et al., 1997), PVP should have strong interactions with activated charcoal due to the sp^2-electronic configuration of carbon rings of the latter. However, the overall DNA yield obtained was consi-derably too low to perform molecular characterization in terms of number of PCR-RAPD reactions from a single extraction. Moreover, optimization of buffer to sample ratio was required which should be 10 to 12 times more (viz., added 1.25 ml of extraction buffer per 25 mg of mature leaf tissue) when compared to the isolation procedure from young leaves which have been described in the following section. For all these reasons, DNA isolation from the mature leaf tissue was not considered for further molecular characterization work eventually leading to exploit of the young leaf tissue of the fennel.

DNA isolation from young leaves

We used the CTAB-DNA isolation procedure (Doyle and Doyle, 1987; Murray and Thompson, 1980) without any additional anti-oxidants (PVP) for the fresh young leaf tissue (Figures 2a to c). In an attempt of using different amount of starting material, it was observed that the ratio of buffer to leaf tissue should always be 5:1 or 4:1 (v/w) in order to obtain good yield with superior quality DNA. From the spectrophotometric measurements, it was calculated that the yield ranged from 1100 to 1900 ng/µl for 200 to 300 mg of young leaf tissue and the purity at OD at A_{260}/A_{280} ranged between 1.8 to 1.9 indicating that the DNA was free of contaminating polysaccharides, polyphenolics and secondary metabolites. The protocol was repeated twice and was applied to 14 different wild fennel samples collected from various sites (Figure 2d). Further, the purified, quantified and diluted DNA was subjected to PCR-RAPD and was found to be very successful in producing scorable, clear amplicons belonging to different populations of fennel (Figures 2e to i). Eventually, the extracted DNA from the fresh young fennel leaf tissue was considered suitable for molecular biology applications with minor modifications in the CTAB-DNA extraction procedure as described in material and methods.

Analysis of PCR-RAPD parameters and amplification conditions

The study was extended in optimizing the PCR-RAPD conditions for further molecular characterization work. DNA isolated by the above method subjected to PCR-RAPD yielded strong and reliable amplicons showing its compatibility for using random decamer primers (Figures 3b, c and 2i). The protocol for PCR-RAPD was optimized by introducing a number of modifications to the original protocol (Williams et al., 1990). Almost all the tested parameters for RAPDs like the concentration of template DNA, PCR buffer, magnesium chloride, *Taq* polymerase, as well PCR cycling conditions such as temperature and time intervals during denaturation, annealing and elongation were optimized which had considerable effect on amplification, banding patterns and reproducibility (Figures 3a to e). The optimized conditions and observed remarks for PCR-RAPD protocol are given in Table 1. Importantly, the present study indicates the necessity of inclusion of detergents [0.1% (v/v) Tween 20, 0.8% (v/v) Nonidet P40] in the 10X PCR buffer yielding consistent and reliable amplicons with an optimized 2.5 mM $MgCl_2$ concentration (Figure 3a). Also, the usage of salt $(NH_4)_2SO_4$ in the 10X PCR buffer over KCl was found to be superior (Figures 3a to c, and 2h and i) substantiating

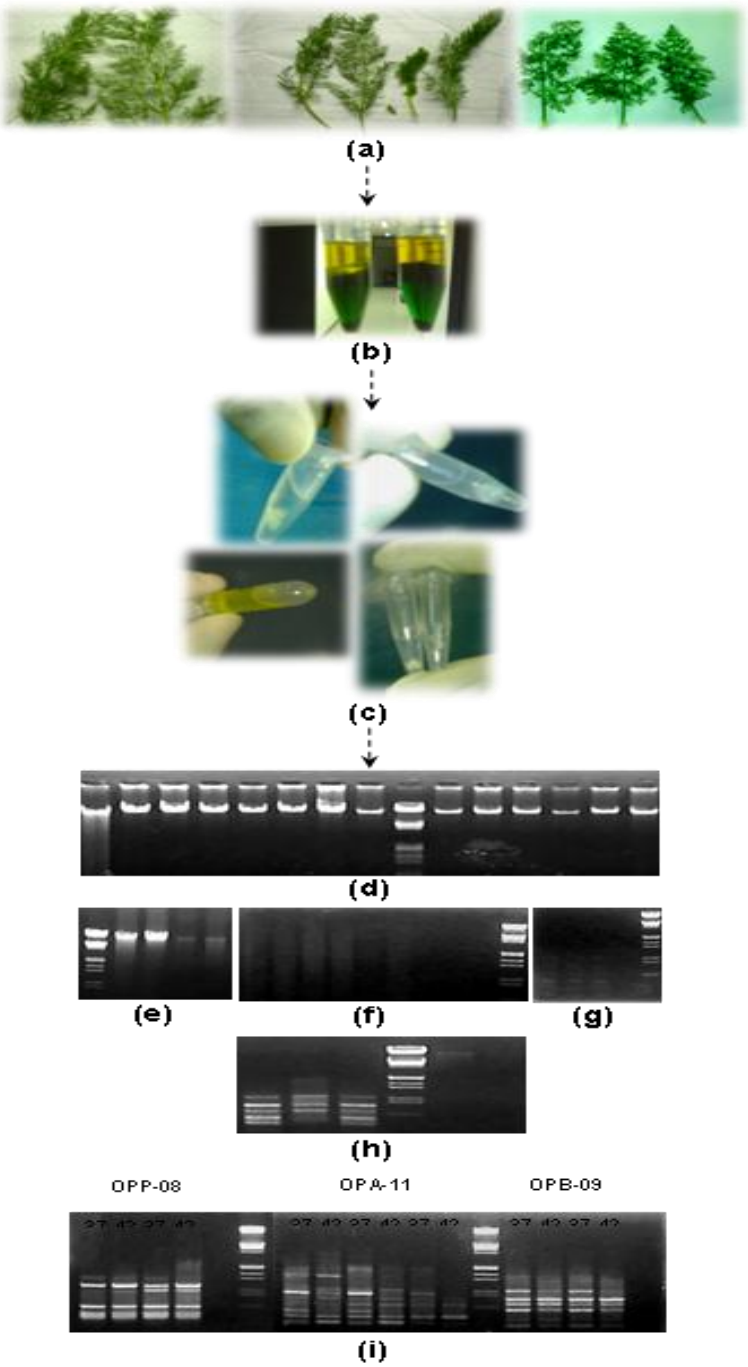

Figure 2. DNA isolated from the young tender leaves and its subsequent PCR-RAPD. (a) Young tender leaves; (b) supernatant with pigments, after chloroform : isoamyl alcohol (24:1) extraction; (c) clumped DNA fibrils and precipitated DNA after addition of ice-cold isopropanol; (d) RNase A digested and purified DNA (14 samples); (e) gel picture showing different dilutions of DNA; (f) smeared PCR-RAPD due to higher template DNA concentration; (g) very faint amplification of PCR-RAPD with lower amount of template DNA; (h) gel picture showing successful amplification after optimized DNA dilution/amount; (i) successful PCR-RAPD after optimized PCR parameters such as DNA template, PCR buffer, $MgCl_2$, *Taq* polymerase, at different annealing temperature (37 and 42°C) with different 10-mer primers and PCR cycling conditions. PCR-RAPD, polymerase chain reaction-random amplification of polymorphic DNA.

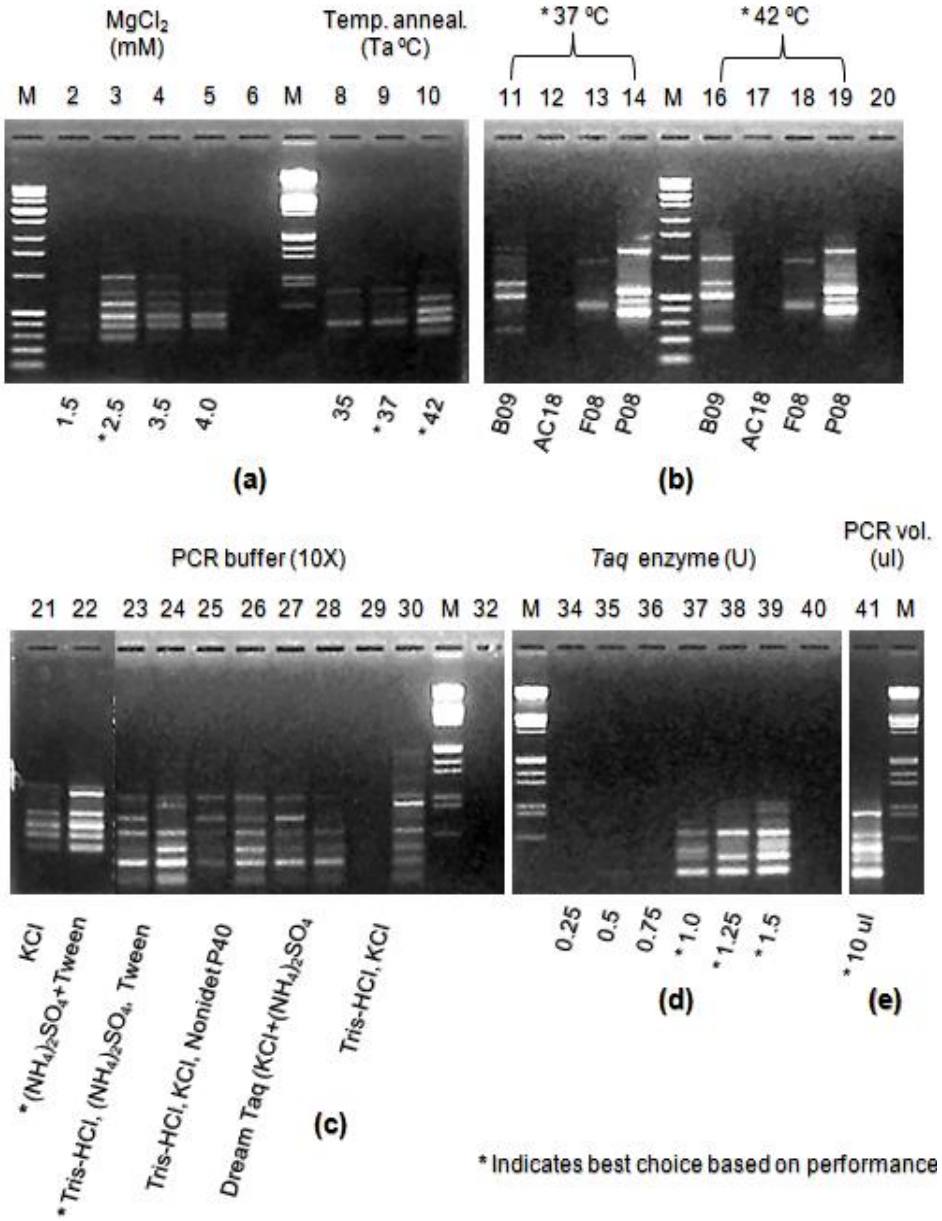

Figure 3. Optimized PCR-RAPD parameters for fennel (*Foeniculum vulgare.* (a) Optimization of $MgCl_2$ and temperature annealing. Lane 1, 1 kb gene ruler; lanes 2 to 5, $MgCl_2$ concentration; 1.5 mM, 2.5 mM, 3.5 mM and 4.5 mM, respectively; lane 6, negative control; lane 7, lambda DNA EcoRI HindIII digest; lanes 8 to 10, temperature annealing at 35, 37 and 42°C with primer A11, respectively. (b) Optimization of 10-mer primers; lanes 11 to 14, B09, AC18, F08 and P08 at 37°C, respectively; lane 15, 1 kb gene ruler; lanes 16 to 19; B09, AC18, F08, P08 at 42°C, respectively; lane 20, negative control. (c) Optimization of PCR buffer (different compositions) with primer A11; lane 21, KCl without any detergent at 42°C; lane 22, $(NH_4)_2SO_4$ with 0.1% (v/v) Tween 20 at 42°C; Lanes 23 and 24: 750 mM Tris-HCl (pH 8.8 at 25 °C), 200 mM $(NH_4)_2SO_4$, 0.1% (v/v) Tween 20, at 37, 42°C, respectively; lanes 25 and 26, 100 mM Tris-HCl (pH 8.8 at 25°C), 500 mM KCl, 0.8% (v/v) Nonidet P40, at 37 and 42°C, respectively; lanes 27 and 28, a commercial, optimized dream Taq buffer - containing KCl and $(NH_4)_2SO_4$ with 20 mM $MgCl_2$, at 37 and 42°C, respectively; lanes 29 and 30, 100 mM Tris-HCl (pH 8.6), 500 mM KCl, 15 mM $MgCl_2$ at 37 and 42°C, respectively; lane 31, lambda DNA EcoRI HindIII digest. lane 32, negative control. (d) Optimization of *Taq* polymerase enzyme; lane 33, lambda DNA EcoRI HindIII digests; lanes 34 to 39, different units of *Taq* 0.25, 0.5, 0.75, 1.0, 1.25 and 1.5 (volume: 25 µl); lane 40, negative control. (e) Optimization of PCR reaction volume; lane 41, 10 µl with proportionate PCR ingredients; lane 42, lambda DNA EcoRI HindIII digest.

Table 1. Optimization of the PCR-RAPD reaction parameters for fennel (*Foeniculum vulgare*).

PCR parameter	Experimented range	Optimum level or condition	Observed remark
DNA concentration (ng)	10, 25, 40 and 50	40 or 50	**Excess:** Existence of smearing **Lower:** Very faint or absence of amplicons (Padmalatha and Prasad, 2006).
***10X PCR buffer (µl)**	**a) $(NH_4)_2SO_4$ with 0.1% (v/v) Tween 20 b) KCl with 0.8% (v/v) Nonidet P40 c) KCl without any detergent d) Optimized buffer (Dream Taq) containing KCl and $(NH_4)_2SO_4$**	**$(NH_4)_2SO_4$ with 0.1% (v/v) Tween 20**	**Performance on the basis of excellence: (a) > (b) > (c, d).**
Magnesium chloride (mM)	1.5, 2.5, 3.5 and 4.0	2.5	**Excess:** non-specific amplification and or poor yield of amplicons. **Lower:** poor, faint amplification (Padmalatha and Prasad, 2006; Harini et al., 2008).
Taq polymerase (Units)	0.25, 0.5, 0.75, 1.0, 1.25 and 1.5	1.25 or 1.5	**Lower:** Failed to amplify. **Excess:** As the concentration increases, showed good amplification with difference in specificity (Padmalatha and Prasad, 2006; Harini et al., 2008).
Deoxynucleotide triphosphates (dNTPs) (mM)	0.2	0.2	Fixed parameter
Primer (10-mer) (pm)	1.5	1.5	Fixed parameter
PCR reaction volume (µl)	10 and 25	10 and 25	**10 µl:** Excellent yield of amplicons **25 µl:** Comparatively more no. of amplicons.
Initial denaturation temperature and time interval (°C) (min)	95 for 5 min 95 for 3 min 94 for 3 min	95 for 3min or 94 for 3 min	Higher than the optimum, resulted in loss of *Taq* activity, lack of reproducibility (Harini et al., 2008).
Annealing temperature (°C)	Tm below 5°C and 35, 37, 40, 42 and 45	37 and 42	Tm below 5°C failed to amplify. Optimum temperatures resulted in difference in specificity with single 10-mer primer. In addition, influenced by choice of PCR buffer as well.
Extension time (min)	1 min 10 sec, 2 min	1 min 10 sec	Gain of *Taq* utility with optimum amplification range.
Number of cycles	30, 35 and 40	35 or 40	The higher the no. of cycles, the better the amplification profile with increased *Taq* concentration (Padmalatha and Prasad, 2006; Harini et al., 2008).

*Bold letter depicts the parameter optimized for the first time in the present study.

"the higher primer specificity and more consistent yield of the specific PCR product over a wide range of $MgCl_2$ concentration as variety of annealing temperatures can be achieved in the buffer with $(NH_4)_2SO_4$ than in the traditional buffer" (MBI, Fermentas Inc, Maryland, USA).

Initially, amplification reactions were performed with <1.0 units of *Taq* polymerase, however, the PCR reaction failed to amplify. Further optimization reactions were carried out at 1.25 to 1.5 units of *Taq* enzyme yielding good amplification profile. RAPD amplification was not reproducible viz., very faint amplification (Figure 2g) or resulted in poor resolution below a certain concentration of DNA, making it difficult to score. Similarly, the higher amount of DNA produced 'smears' (Figure 2f). Hence, a series of dilutions (Figure 2e) were made to verify the best range of DNA concentration in order to get the optimum amplification profile. Subsequently the PCR volume was also optimized (10 and 25 µl) with reduction in the template DNA (µl). PCR trials were also undertaken with different concentrations of magnesium chloride ($MgCl_2$) (Table 1) keeping all other parameters constant. $MgCl_2$ at 2.5 mM was proved to be best (Figure 3a). In general, primer annealing in most PCR-RAPD reactions were carried out widely at 35 to 55°C. In our PCR trials, the annealing temperature of 37 and 42°C was determined by three different trials by using gradient PCR. An annealing temperature 5°C below the Tm of the 10-mer primer, failed to amplify while the optimized temperatures resulted in difference in specificity with single 10-mer primer at 37 and 42°C (Table 1). In addition, annealing behaviour of the individual primer was influenced by the choice of 10X PCR buffer as well. Significant differences in specificity and band intensity were found among PCR products obtained from PCR programs run at 37 and 42°C (Figures 3b, c and 2i) with different PCR buffers. Notably, the determination of the annealing temperature will also depend on the complexity of the genomic DNA and GC% of the decamer primer. Hence, we propose that it is required to optimize the individual random primers for at least two different annealing temperatures viz., 37 and 42°C and then make out the scores in comparison to the two RAPD profiles obtained per primer for an individual. Genomic DNA denaturation is a critical step in PCR-RAPD amplification reactions. To ensure the entire DNA molecules have denatured, we attempted two different temperatures and time intervals (Table 1) and found that 94 or 95°C for 3 min worked best. This result could probably be attributed to the fact that *Taq* polymerase (viz., for routine lab work) has a limited lifespan at high temperatures (Gelfand, 1989). For amplification of PCR products shorter than 1.2 kb, an extension time of 45 s was sufficient but longer PCR products required an extended time of 1 min 10 s at which fragments as large as 1.5 to 2 kb were amplified (Figures 3 and 2i). In conclusion, the optimized DNA extraction method and PCR reaction conditions produced clear, scorable amplicons suitable for RAPD analysis (Figures 3a to e and 2i). The size of the amplified fragments ranged from 150 - 2,000 bp for a single decamer primer and varies upon different primers. In our experiments, the PCR parameters and reaction conditions were optimized considerably for fennel (*F. vulgare*) in order to obtain reproducible profiles. Moreover, this study clearly shows that it is preferable to work with fresh young leaves over the matured or stored leaf tissue since the age of the plant (developmental stage/seasonal condition) greatly influences/hinders the objective of the present study.

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