

Full Length Research Paper

A simple and rapid DNA extraction method from leaves of grapevine suitable for polymerase chain reaction analysis

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The genomic grapevine (*Vitis vinifera* L.) DNA extraction is difficult because of secondary metabolites that interfere with DNA isolation procedures and subsequent applications. We developed a simple, rapid and efficient method for the extraction of genomic DNA from asymptomatic and pathogen-infected grape leaves. The protocol reported, based on a modified cetyl trimethylammonium bromide (CTAB) extraction procedure, allowed the rapid DNA extraction from little amounts of leaf material without employment of liquid nitrogen for initial tissue grinding. The protocol included polyvinylpyrrolidone (PVP) to bind phenolic compounds, β -mercaptoethanol to inhibit the oxidation of polyphenols, and a high concentration of NaCl (2.5 M) to increase the solubility of polysaccharides, thus reducing their co-precipitation with DNA. Final DNA solution did not contain polysaccharides, polyphenols and other major contaminants. The purity of genomic DNA was confirmed by $A_{260/280}$ and $A_{260/230}$ ratios calculated from the spectrophotometric readings. In addition, the quality of the DNA extracted from asymptomatic, *Oidium tuckeri*- and *Plasmopara viticola*-infected leaves of *V. vinifera* L. was evaluated in polymerase chain reaction (PCR) analyses by using different set of primers to be able to amplify vegetal, fungal and bacterial DNA.

Key words: *Vitis vinifera* L., DNA extraction, PCR, fungi, bacteria.

INTRODUCTION

Grapevine (*Vitis vinifera* L.), a member of the *Ampelidaceae* or *Vitaceae* family, is one of the oldest and most important perennial crops in the world. Recently, this plant has been used for gene mapping (Kikkert et al., 2005; Akkurt et al., 2007; Troglio et al., 2007), genetic transformation (Wang et al., 2005), and DNA fingerprinting (Adam-Blondon et al., 2004; Di Gaspero et al., 2007).

However, molecular markers require a fair amount of high purity DNA and it is often not easy to separate DNA from naturally occurring plant cell contaminants.

The genomic grapevine DNA extraction is difficult, especially when young leaves are unavailable. In fact, it is known that mature grapevine leaves contain high quantities of secondary metabolites, such as polysaccharides, polyphenols and tannins (Lodhi et al., 1994; Hanania et al., 2004). In addition, abiotic stress such as water and nutritional deficiencies or pathogen infection can significantly enhance the biosynthesis and accumulation of these secondary compounds (landolino et al., 2004). These contaminants have also been reported to cause difficulty in DNA purification in other plant species (Murray and Thompson, 1980; Katterman and Shattuck, 1983; Fang et al., 1992; Aljanabi et al., 1999). When plant

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Abbreviations: CTAB, Cetyl trimethylammonium bromide; EDTA, ethylenediaminetetraacetic acid; PCR, polymerase chain reaction; PVP, polyvinylpyrrolidone.

cells are destroyed, these cytoplasmic contaminants come into contact with nuclei and other organelles, representing a major obstacle in DNA purification (Loomis, 1974; Fang et al., 1992; Aljanabi et al., 1999; Hanania et al., 2004). In particular, polyphenolic compounds can irreversibly bind proteins and nucleic acids to form high-molecular weight complexes (Porebski et al., 1997), whereas polysaccharides tend to co-precipitate with DNA (landolino et al., 2004) making the DNA viscous and un-amplifiable in polymerase chain reaction (PCR). Therefore, these contaminants must be eliminated during DNA isolation. Although, several DNA extraction protocols have been described for plants containing high concentrations of secondary metabolites, the most of these require a large amount of plant tissue to be ground in liquid nitrogen (Busconi et al., 2003; Nazhad and Solouki 2008). In addition, liquid nitrogen is unavailable in many regions of the world. In this study, we describe a simple, rapid and efficient method to extract high quantities of quality genomic DNA from little amounts of asymptomatic grape leaves and grape leaves infected by some pathogens. The method involves a modified cetyl trimethylammonium bromide (CTAB) extraction procedure (Doyle and Doyle, 1990). Moreover, we demonstrate the usefulness of the extracted DNA for PCR-based techniques.

MATERIALS AND METHODS

Plant material

Thirty-six grapevine leaves, collected from plants cv. Insolia of two Sicilian vineyards located in Palermo (38°06'N, 13°21'E; elevation 14 m) and Trapani (37°47'N, 12°34'E; elevation 12 m), were used for genomic DNA extraction. Six plants for each vineyard, two asymptomatic, two infected by *Oidium tuckeri* and two infected by *Plasmopara viticola*, were sampled; three leaves for each plant were collected. All samples were frozen in dry ice at the time of survey and then stored at -80°C until DNA extraction.

Moreover, three leaves of two certified pathogen-free plants, purchased from the nursery "Cuciti Vivai Trinacria Vitis", were also used for DNA extraction.

Solutions

Extraction buffer consisted of 2% CTAB, 100 mM Tris-HCl (pH 8.0), 25 mM ethylenediaminetetraacetic acid (EDTA) (pH 8.0), 2.5 M NaCl, 2% polyvinylpyrrolidone (PVP), and 1% β -mercaptoethanol. In addition, chloroform:isoamyl alcohol (24:1, v/v), isopropanol, 70% ethanol, TE buffer [10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0)], and RNase A (10 mg/ml) were prepared and stored.

DNA extraction protocol

The frozen leaves were taken out of the freezer and quickly ground to a fine powder with mortar and pestle (frozen rapidly at -80°C). Powders (200-300 mg) were immediately transferred to a 1.5 ml microfuge tube containing 700 μ l of preheated (60-65°C) CTAB buffer. The tubes were shaken and incubated at 65°C for 60 min in a water bath, mixing by inversion 3-4 times during incubation.

Samples were centrifuged at 10000 rpm for 10 min at 4°C and the aqueous phase was gently transferred into a new tube. Equal volume of chloroform:isoamyl alcohol (24:1) was added and mixed by inversion. Samples were centrifuged at 10000 rpm for 10 min at 4°C and the aqueous phase was transferred into a new tube. Equal volume of isopropanol (-20°C) was added to separate the DNA; samples were mixed and incubated at -20°C for 30 min. Precipitated DNA was centrifuged at 14000 rpm for 10 min at 4°C and supernatant was discarded. DNA pellets were washed with 500 μ l of 70% ethanol (-20°C) and centrifuged at 14000 rpm for 5 min at 4°C. The resulting DNA pellets were air-dried at room temperature and dissolved in 200 μ l of TE buffer. RNase A was added to each sample (1/100 μ l DNA sample) and was incubated at 37°C for 1 h. Samples were stored at -20°C.

Quantity and purity of DNA

Quantity and purity of the DNA extracted from all samples were checked by NanoDrop 1000 Spectrophotometer (Thermo Scientific). The absorbance ratios A_{260}/A_{280} for protein contamination and A_{260}/A_{230} for the presence of polyphenolic / polysaccharide compounds were used.

PCR analysis

In order to test the ability of amplification of extracted genomic DNA and to determine possible inhibitory materials which may interfere with the reactions, PCR analysis of DNA from all samples were carried out.

To analyze the quality of the plant DNA, grapevine cultivar-specific SCAR (sequence characterized amplified regions) primers OPF16Fw (5'-GGAGTACTGGTTCCTACTAG-3') and OPF16Rv (5'-GGAGTACTGGCCTTACTC-3') (Vidal et al., 2000) were used. The amplification reaction was performed in a total reaction volume of 25 μ l containing 50 ng of DNA template, 100 μ M of dNTPs, 0.2 μ M of each primer, 0.5 U of Taq DNA Polymerase (GoTaq, Promega, USA) and 1X GoTaq buffer (1.5 mM $MgCl_2$, Promega). A negative control (PCR mixture without DNA) was included in all PCR experiments. The amplification reaction was carried out in a Thermocycler T1 (Biometra) as follows: initial denaturation at 94°C for 2 min, 45 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, extension at 72°C for 2 min and final extension at 72°C for 7 min.

To amplify the internal transcribed spacer regions (ITS1-5.8S-ITS2) of rRNA gene from fungi, primers ITS1F (fungus specific: 5'-CTGGTCATTTAGAGGAAGTAA-3') (Gardes and Bruns, 1993) and ITS4 (universal: 5'-TCCTCCGCTTATTGATATGC-3') (White et al., 1990) were used. The reaction volume (40 μ l) contained 50-100 ng of DNA template, 2 mM of $MgCl_2$, 0.2 mM of dNTP, 0.3 μ M of each primer, 0.5 U of Taq DNA Polymerase (Dream Taq, Fermentas, Italy) and 1X Dream Taq buffer (Fermentas). As positive control *Acremonium byssoides* strain A21, endophyte in asymptomatic grape leaves (Burruano et al., 2008), was used. The amplification program consisted of one initial denaturation at 95°C for 2 min, followed by 35 cycles of 95°C for 30 s, 55°C for 20 s, 72°C for 1 min and a final extension at 72°C for 5 min.

To amplify the 16S rRNA gene from bacteria, universal primers fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and rD1 (5'-AAGGAGGTGATCCAGCC-3') (Weisburg et al., 1991) were used. The 50 μ l PCR mixture contained 50-100 ng of DNA template, 2.5 mM of $MgCl_2$, 0.25 mM of dNTPs, 0.2 μ M of each primer, 2.5 U of Taq DNA Polymerase (Dream Taq, Fermentas) and 1X Dream Taq buffer (Fermentas). As positive control *Bacillus subtilis* strain AG1, isolated from grape wood infected by "esca" syndrome (Alfonzo et al., 2009), was used. The PCR was performed under the following conditions: 95°C for 3 min; 30 cycles of 94°C for 1 min, 54°C for 45

Table 1. Evaluation of extracted DNA according to spectral absorbance ratios (A₂₆₀/A₂₈₀ and A₂₆₀/A₂₃₀) and final concentration (ng/μl).

Sample leaf	A ₂₆₀ /A ₂₈₀	A ₂₆₀ /A ₂₃₀	Cc (ng/μl)
Asymptomatic	1.89 ± 0.02	2.18 ± 0.01	270.0 ± 22.2
<i>Oidium tuckeri</i> -infected	1.82 ± 0.02	2.08 ± 0.02	246.8 ± 20.3
<i>Plasmopara viticola</i> -infected	1.80 ± 0.01	2.02 ± 0.01	205.2 ± 18.2
Certified pathogen-free	1.87 ± 0.01	2.15 ± 0.01	263.0 ± 19.8

Values are mean ± SE (standard error).

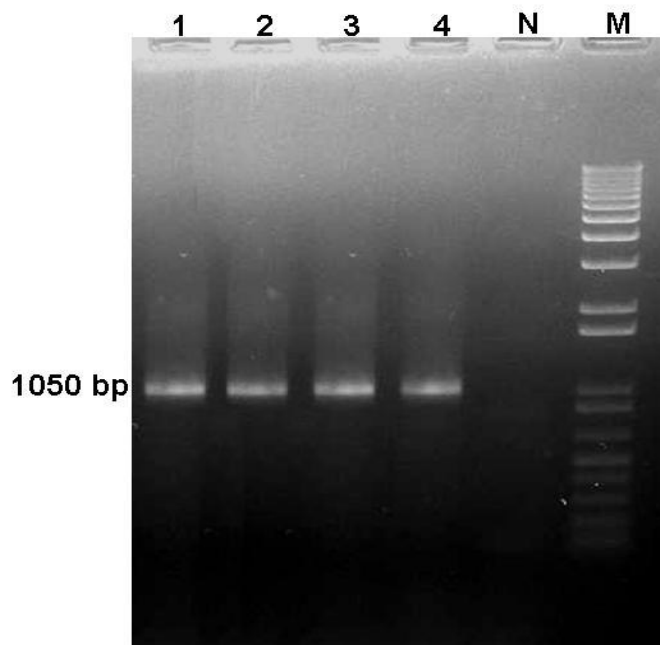


Figure 1. PCR products obtained using the primers OPF16Fw and OPF16Rv from asymptomatic leaf (Lane 1), *Oidium tuckeri*-infected leaf (Lane 2), *Plasmopara viticola*-infected leaf (Lane 3) and free-pathogen leaf (Lane 4). N: Negative control without DNA. M: Molecular marker (1 Kb plus DNA ladder, Invitrogen, USA).

s, 72°C for 2 min; 72°C for 7 min. Five microliters of all PCR products were analyzed by electrophoresis in a 1.5% agarose gel stained with SYBR Green I nucleic acid stain and visualized under UV light.

RESULTS AND DISCUSSION

Grapevine contains high concentrations of polysaccharides, polyphenols, tannins and other secondary metabolites (Lodhi et al., 1994; Hanania et al., 2004). These compounds may hamper the DNA isolation processes and subsequent analysis such as DNA restriction, amplification, cloning, genomic DNA blot hybridization, and genomic DNA library construction (Pandey et al., 1996; Khanuja et al., 1999; Li et al., 2007; Barzegari et al., 2010). Therefore, these contaminants must be eliminated during DNA isolation.

The modified extraction method (Doyle and Doyle, 1990) reported in this study proved to be effective. We have obtained, in a few hours, high yields of DNA from little amounts of grapevine leaf tissues (0.2 to 0.3 g), avoiding the tedious grinding of each specimen in liquid nitrogen, unlike the standard method that started from 0.5 to 1 g of leaf material and involved the use of liquid nitrogen. This method does not require lyophilization of sample and expensive laboratory material. In agreement with previous reports (Fang et al., 1992; Lodhi et al., 1994), PVP and β-mercaptoethanol were absolutely necessary to remove polyphenols from mature and damaged leaf tissues. Moreover, the use of a higher molar concentration (2.5 M) of NaCl, compared to the original protocol (1.4 M), enabled elimination of the polysaccharides. It should be emphasized that the amount of leaf tissue used for DNA extraction should not exceed 300 mg per sample. Otherwise, it is difficult to effectively remove contaminants even when PVP, β-mercaptoethanol and NaCl are used in later steps.

The spectrophotometer analysis for A₂₆₀/A₂₈₀ and A₂₆₀/A₂₃₀ resulted in 1.80 to 1.89 and >2 on average, respectively indicating that the extracted DNA was free from proteins and polyphenolic/polysaccharide compounds (Table 1). The average final concentration of DNA ranged from 205 to 270 ng/μl (Table 1).

The purity and the quality of the extracted DNA were confirmed by PCR analysis. In fact, amplifiable DNA was obtained from all tested leaves. In particular, using grapevine cultivar-specific SCAR primers, a fragment of 1050 bp was amplified (Figure 1). All the samples also exhibited successful amplification of ITS regions of rRNA gene from fungi and 16S rRNA gene from bacteria, as demonstrated by clear bands of ~600 bp (Figure 2) and ~1600 bp (Figure 3) obtained, respectively. The detection of fungal DNA also in leaves from certified pathogen-free plants and in asymptomatic leaves is probably due to the presence of fungal endophytes, as reported in *V. vinifera* by Mostert et al. (2000) and Burruano et al. (2008). Moreover, endophytic bacteria in grapevine leaf tissues have also been reported by Bulgari et al. (2009) and Lo Piccolo et al. (2010); this explains the presence of bacterial DNA in all sampled leaves. Furthermore, the DNA could be used for denaturing gradient gel electrophoresis (DGGE) analysis to study different bacterial and fungal compositions between asymptomatic and

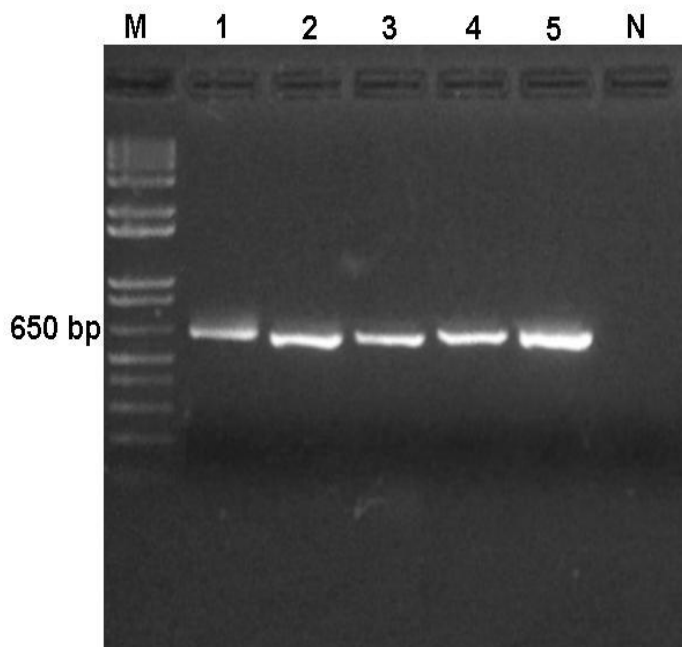


Figure 2. Amplification of ITS regions of rRNA gene from asymptomatic grapevine leaf (Lane 1), *O. tuckeri*-infected leaf (Lane 2), *P. viticola*-infected leaf (Lane 3), free-pathogen leaf (Lane 4) and *Acremonium byssoides* strain A21 (Lane 5). N: Negative control without DNA. M: Molecular marker (1 Kb plus DNA ladder, Invitrogen).

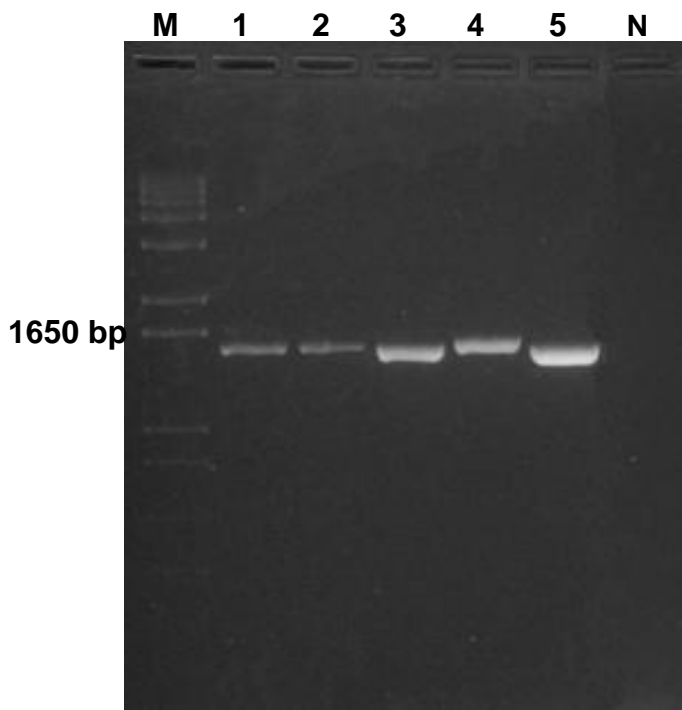


Figure 3. Amplification of 16S rRNA gene from asymptomatic grapevine leaf (Lane 1), *O. tuckeri*-infected leaf (Lane 2), *P. viticola*-infected leaf (Lane 3), free-pathogen leaf (Lane 4) and *Bacillus subtilis* strain AG1 (Lane 5). N: Negative control without DNA. M: Molecular marker (1 Kb plus DNA ladder, Invitrogen).

symptomatic grape leaves.

Conclusion

The protocol reported in this study, that includes the use of PVP, β -mercaptoethanol and a high concentration of NaCl (2.5 M), without the tedious employment of liquid nitrogen allows the rapid and simple isolation of quality genomic DNA from little amounts of leaf tissues of *V. vinifera* L., suitable for PCR amplification and subsequent molecular analysis.

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