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New simple and efficient method of DNA isolation from pear leaves rich in polyphenolic compounds

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Summary: This study aimed to establish a new protocol for DNA isolation from the *Pyrus* genus to get high quality DNA that is suitable for the generation of molecular markers, such as RAPD and AFLP. This method is based on modified CTAB extraction procedure (Aldrich & Cullis, 1993). For isolation of high-quality DNA we used copper (II) acetate treatment that enabled fixation and removing of tannins, present in abundance in *Pyrus*. DNA yield from this procedure is high (up to 2.5 $\mu g \cdot \mu l^{-1}$). DNA is completely digestible with restriction endonucleases and amplifiable in the PCR, indicating freedom from common contaminating polyphenolic compounds.

Key words: pear DNA isolation, polyphenolic compounds, tannins, copper (II) acetate, *Pyrus*

Introduction

The isolation of high-quality DNA is prerequisite for any molecular biology work because contaminants such as proteins, polyphenols and polysaccharides may interfere with enzymes, such as restriction enzymes (in blotting techniques) and *Taq* polymerase in PCR (polymerase chain reaction) (*Ausubel* et al., 1994). Leaves, twigs, flowers, fruits and seeds of pear contain phenolic compounds (*Friedrich,* 1957, 1958a, 1958b), to which belong among others tannins and phenolic glucosides, e.g. arbutin. During preparation of plant material to extraction and during extraction of DNA we observed browning of tissue and supernatant. This phenomenon depends on genotype and did not allow to isolate high quality DNA with standard methods. Browning during the processing has generally been interpretated in terms of oxidation of caffeoylquinic acid and catechins by catecholase activity of pear polyphenol oxidase (*Tate* et al., 1964; *Halim & Montgomery*, 1978; *Amiot* et al., 1992; *Veltman* et al., 1999). During homogenization of plant material, polyphenols are released from vacuoles and they then react rapidly with cytoplasmic enzymes (*Sarwat* et al., 2006). The phenols covalently bind to proteins and DNA, giving the DNA a brown colour and making it useless for most research applications (*Katterman & Shattuck*, 1983; *Guillemaut & Maréchal-Drouard*, 1992, *Aljanabi* et al., 1999). Polyphenol contamination of DNA makes it resistant to restriction enzymes (*Katterman & Shattuck*, 1983). Also pear tissues infected by the bacteria develop a dark brown or black colour which contributes to the charred appearance characteristic of the disease. The colour results from the oxidation of phenolic compounds produced during the breakdown of arbutin, a phenolic β-glucoside found in pears (*Kerppola* et al., 1987). Arbutin can be hydrolyzed by β-glucosidase enzymes to release hydroquinone. Because the arbutin concentration in pears is approximately 10,000 ppm and free hydroquinone concentrations ranging up to 8,000 ppm have been reported in damaged pear tissue (*Smale & Keil*, 1966), several groups of investigators have suggested that hydroquinone plays a role in plant resistance to fire blight disease (*Chatterjee* et al., 1969; *Hildebrand & Schrotch*, 1963; *Smale & Keil*, 1966). Hence for the first time we attempted to use copper (II) acetate in the extraction process of DNA in order to remove contaminants. Copper (II) acetate is reagent successfully applied for quantitative determining of tannins according to the *Pharmacopea polonica* (1995).

Materials and methods

Pear genomic DNA was isolated from the leaves of young seedlings that differ in their susceptibility to fire blight after experimental inoculation with *Erwinia amylovora*. Seedlings were 50 cm high. Protocol of DNA extraction, based on Aldrich and *Cullis* method (1993), has been modified and copper (II) acetate solution has been applied.

Reagents and buffers

- CTAB (hexadecyltrimethylammonium bromide) extraction buffer:
  2% CTAB
  2% PVP-40 (polivinylpolypyrrolidone), average mol wt 40000
  5 M NaCl
  20 mM EDTA (ethylenediaminetetraacetic acid)
  100 mM Tris-Cl, pH 8.0
  2% β-mercaptoethanol

Dissolve CTAB and other reagents by heating to 65°C. Add 2% β-mercaptoethanol just before use.

- Chloroform: isoamyl alcohol (24:1)
- 100% isopropanol
- 0.1 n copper (II) acetate solution
- 99.8% ethanol
- wash buffer:
  76% ethanol
  10 mM ammonium acetate
- 10 mg•ml^{-1} RNase A

Protocol

- Collect young leaves on ice and as soon as possible freeze in liquid nitrogen (N_2). Lyophilize them and store till DNA isolation (we stored at -20 °C).
- 100 mg of lyophilized tissue grind into a fine powder using pestle in 2.0 ml centrifuge tubes in the presence of liquid nitrogen.
- Add 900 µl of preheated extraction buffer (65 °C). Stir with pipette tip, then shake vigorously with Vortex and immediately place back at 65 °C.
- Incubate at 65 °C for 1 or more hours. Longer incubations result in higher yield and cleaner, whiter pellets (*Aldrich & Cullis*, 1993).
- Extract with an equal volume of chloroform: isoamyl alcohol (24:1) and mix gently by inverting the tubes for 5 minutes to form an emulsion.
- Centrifuge the samples for 10 minutes at room temperature at 12000 rpm to separate phases.
- Transfer the top aqueous phase to a new 1.5 ml microcentrifuge tube.
- Add 50 µl of copper (II) acetate (cupric acetate) solution. Mix gently by inverting the tubes for 3 minutes.
- Centrifuge the samples for 5 minutes at room temperature at 12000 rpm.
- Transfer the top aqueous phase to a new 1.5 ml microcentrifuge tube.
- Precipitate the DNA with 2/3 volume of isopropanol.
- Spin the precipitated nucleic acids by centrifuging at room temperature for 10 minutes at 12000 rpm.
- Discard the supernatant and wash the pellet twice with wash buffer. CTAB is soluble in ethanol and residual amounts are removed in this step (*Aldrich & Cullis*, 1993).
- Air-dry the pellets and resuspend in ultra pure water.
- Add 3 µl of RNase A and incubate 30 minutes at 37 °C.
- Bring the dissolved nucleic acids to 2 M NaCl. During ethanol precipitation of nucleic acids from 2M NaCl, polysaccharides remain dissolved in the ethanol (*Fang* et al., 1992). The freer the nucleic acids are from contaminants, the easier the pellet is to resuspend (*Aldrich & Cullis*, 1993).
- Re-precipitate using 2 volumes of 99.8% ethanol. Incubate at -20 °C for 20 minutes. Centrifuge the samples for 20 minutes at 4 °C at 10000 rpm.
- Discard the supernatant and wash the pellet with 500 µl of 70% ethanol, air-dry the pellet and resuspend in ultra pure water. Washing in 70% ethanol removes residual NaCl and CTAB.

The purity of the DNA was determined through spectrophotometric analyses. The A260/A280 ratio averaged 1.84 and ranged from 1.66 – 2.03. DNA yield from this procedure is high (up to 2.5 µg•µl^{-1}).

RAPD (Random Amplification of Polymorphic DNA) analysis

DNA extracted by our modified method was subjected to RAPD analysis to verify its amplificability. Analysis was performed in a reaction volume of 25 µl containing 1 x *Taq* Polymerase Buffer, 3 mM $MgCl_2$, 1.25 U of *Taq* DNA Polymerase (MBI Fermentas), 200 µM dNTPs (MBI Fermentas), 200 µM of random decamer primer (5'-GTGATCGCAG-3'), 200 µg•ml^{-1} BSA and total genomic DNA. Amplifications were carried out using a DNA thermal cycler (Mastercycler, Eppendorf) with the following parameters: 4 min at 94 °C, 45 cycles of 15 sec at 94 °C, 10 sec at 32 °C, and 10 sec at 72 °C, and finally 7 min at 72 °C, followed by cooling to 4 °C. The products were size fractionated on 1.5% agarose gel and visualized under UV light after ethidium bromide staining.

Results and Discussion

Hitherto reported methods of DNA isolation including those of *Aldrich & Cullis* (1993) and *Lodhi* et al. (1994) proved unsuccessful and unreliable for *Pyrus* as the DNA obtained was brown in appearance. This may be due to high endogenous levels of polysaccharides, phenolics and other organic constituents that interfere with DNA isolation and purification. In many protocols the addition of PVP has been used to isolate of genomic DNA from polyphenol-rich plants to remove polyphenols from leaf tissues (*Aljanabi* et al., 1999; *Chaudhry* et al., 1999; *Doyle & Doyle*, 1987; *Howland* et al., 1991; *Lodhi* et al., 1994; *Kim* et al., 1997; *Rogers & Bendich*, 1985; *Sarwat* et al., 2006). The PVP or PVPP (polivinylpolypyrrolidone) efficiently forms complex hydrogen bonds with polyphenolic compounds and alkaloids that can affect DNA quality (*Cremer & Van de Walle*, 1985; *Gegenheimer*, 1990; *Davidsen*, 1995) and can be separated by centrifugation (*Maliyakal*, 1992). This is also reported to improve stability of enzymes. However in our investigations brown colour of precipitated *Pyrus* DNA was not lost on supplementation with PVP in the extraction process. Only when copper (II) acetate has been applied, fixation and removing of tannins were possible. Generation of RAPD markers was possible using DNA obtained in this way in opposite to DNA extracted without copper (II) acetate (results depicted in *Figure 1*). Also DNA obtained by our modified protocol has been successfully used in our laboratory for AFLP (Amplified Fragment Length Polymorphism) (results not yet published). In the case of two genotypes AFLP analysis was applied for DNA isolated with- and without copper (II) acetate. DNAs were

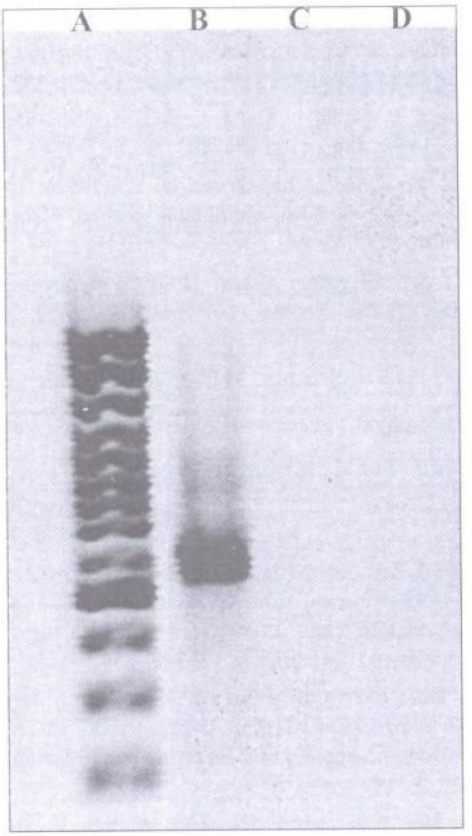

Figure 1. Products of RAPD analysis from different isolations: lane **A** – ladder, lane **B** – DNA extracted with copper (II) acetate, lane **C** and **D** – lack of products – DNA extracted without copper (II) acetate.

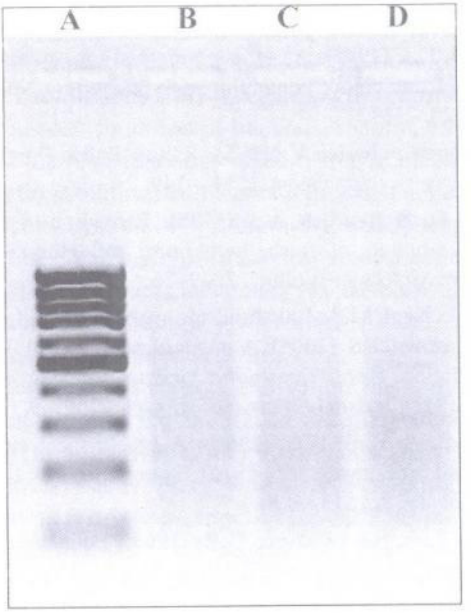

Figure 3. Products of pre-amplification: lane **A** – ladder, lanes **B**, **C**, **D** – visible DNA smear between 100 – 700 bp.

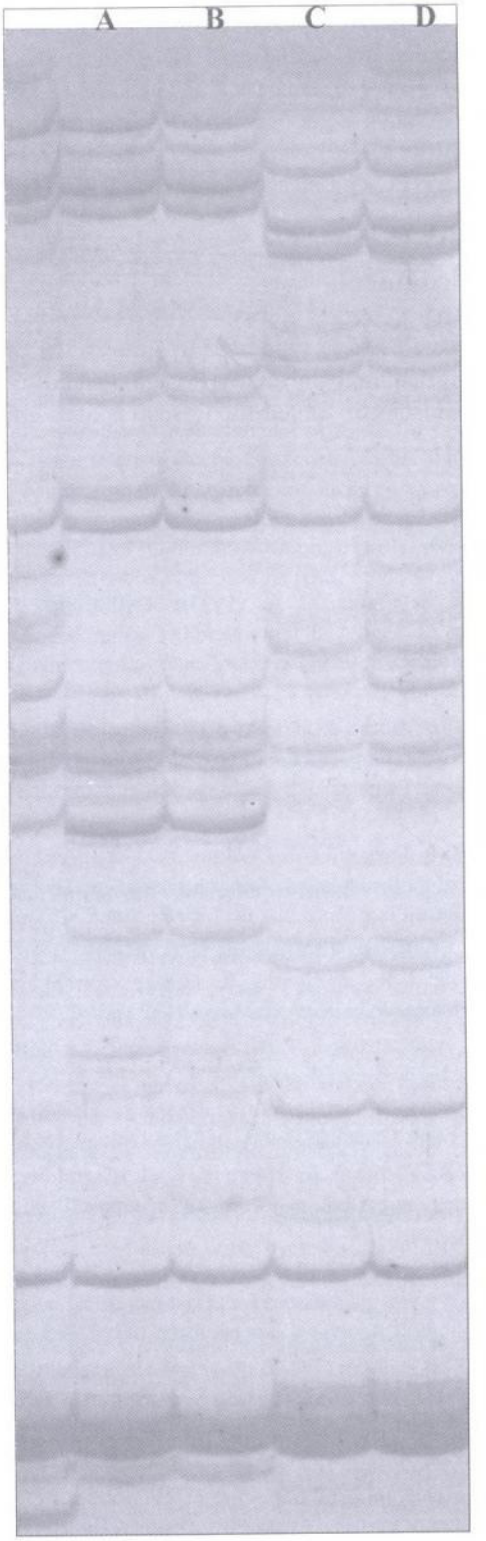

Figure 2. DNA band-pattern of two genotypes. In each case two methods of extraction were applied: with- and without copper (II) acetate. Genotype I: lane A – DNA extracted with copper (II) acetate, lane B – DNA extracted without copper (II) acetate. Genotype II: lane C – DNA extracted with copper (II) acetate, lane D – DNA extracted

extracted from the leaves of trees not infected with pathogen *Erwinia amylovora* and characterised by low contamination of polyphenolic compounds. It enabled us to apply method with- and without copper (II) acetate. Any differences in bands pattern of DNA extracted with both methods were observed (*Figure 2*). Thus it indicates that restriction endonucleases in both cases bound specifically to recognition sequence and cleaved double-stranded DNA at specific sequence of nucleotides. Products of

digestion with *Eco*RI and *Mse*I restriction enzymes following pre-amplification are visible as a DNA-smear ranging from 100–700 bp (*Figure 3*). We have thus established a new suitable method for isolation of good quality DNA from *Pyrus*, a problem tree material for DNA isolation, that can be applicable to both restriction, and PCR based analyses such as RAPD and AFLP.

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