JOURNAL OF PLANT PROTECTION RESEARCH
Vol. 43, No. 4 (2003)

IMPROVED METHOD OF ISOLATION OF TOTAL NUCLEIC ACIDS FROM HOP PLANTS AND GRAPEVINE BEFORE THE RT-PCR BY ADDITION OF POLYVINYLPOLYPYRROLIDONE

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Accepted: November 17, 2003

Abstract. In performed experiments, insoluble polyvinylpolypyrrolidone, PVPP as an additive to the extraction buffer was used for isolation of total nucleic acids from hop plants and grapevine in order to obtain templates useful for detection of HLVd and HSVd by means of RT–PCR. Addition of 2% of PVPP to the original GTC buffer (Chomczynski and Sacchi, 1987) appeared to be the most favorable. Due to PVPP addition, the protocol of extraction of nucleic acids was simplified by shortening of isolation time and reduction of expenses. However, application of the simplified method for obtaining of templates that guaranteed full repeatability of test results was limited to the spring and early summer season.

Key words: PVP, PVPP, polyvinylpolypyrrolidone, RT-PCR, reverse transcription-polymerase chain reaction, HLVd, HSVd

INTRODUCTION

Polymerase chain reaction (PCR) and reverse transcription-polymerase chain reaction (RT-PCR) have been widely used in plant protection studies for detection and identification of such pathogens as viruses or viroids. In most cases purity of nucleic acids used as templates for PCR or RT-PCR is a crucial factor on which positive result of the reaction depends. Such substances as polysaccharides and various products of secondary metabolic pathways, particularly phenolic derivatives, resins and tannins may inhibit with different efficiency or completely block activity of the enzymes participating in these reactions.

A high concentration of polysaccharides in the material can be confirmed just during extraction of nucleic acids, when due to a high viscosity mixture is dense, sticky and difficult for pipetting. Moreover, polysaccharides block thermostable polymerase so, amplification of nucleic acids during PCR appears impossible (Fang et al. 1992).

In most species of higher plants polyphenolic derivatives are very common. They are easy oxidized and form covalently bound quinones (Loomis 1974; Baxter 1997). In this configuration they form complexes with nucleic acids making them useless in such processes as reverse transcription or amplification during PCR (Su and Gibor 1988; Wan and Wilkins 1994; Soni and Murray 1994).

Polyphenolics have occurred in various concentrations in different organs of higher plants. Moreover, their concentration increases with age of cells producing them. There are a lot of methods for extraction of nucleic acids from various plant species that avoid further inhibition of reactions with nucleic acids participation (Chomczynski and Sacchi 1987; Tesnierre and Vayda 1991; Maliyakal 1992; Wang and Cutler 1993; Levy et al. 1994; Lodhi et al. 1994; Staub et al. 1995; Peterson et al. 1997; Porebski et al. 1997; Zhang et al. 1998; Salzman et al. 1999, Morris et al. 2001).

The main aim of this work was to modify guanidine-thiocyanate method (Chomczynski and Sacchi 1987) to get quick, inexpensive and effective procedure for isolation of nucleic acids that could use as templates in the RT-PCR test for detection of two viroids namely, *Hop latent viroid* (HLVd) and *Hop stunt viroid* (HSVd). Nucleic acids were isolated from plants such as hop or grapevine, classified to so called difficult category.

MATERIAL AND METHODS

Total nucleic acids in experiment performed were isolated from disks of diameter of 7 mm cut out from the leaf laminae. Samples were taken from old leaves (the base of steam) in the middle of June. These leaves contained a high concentration of secondary metabolites.

For the extraction of total nucleic acids simplified method of Chomczynski and Sacchi (1987) was used. Samples were homogenized in 1.5 mL Eppendorf tubes using 400 μL of lysing buffer, GTC. The homogenate was incubated in darkness for 1 h at room temperature. After addition of 0.1 volume of 2 M sodium acetate (pH 4.0) and equal volume of phenol as well as 400 μL of chloroform samples were vortexed and then centrifuged for 5 min. at 20°C, 11 000 rpm (centrifuge 1K15, Sigma). Extraction was repeated using mixture of chloroform – isoamyl alcohol (49:1).

After centrifugation the supernatant was collected and equal volume of cold (–20°C) isopropanol was added. The obtained mixture was kept at –20°C for 20 min. Precipitated nucleic acids were centrifuged at 11000 rpm, 4°C for 15 min. Precipitates were dried using a water aspirator, double washed with 200 μL of cold (–20°C), 96% ethanol that was also similarly removed. After drying, precipitates of nucleic acids were dissolved in 50 μL of DEPC treated water and stored at –20°C until use for RT-PCR.

Due to significant simplification of the original procedure of Chomczynski and Sacchi (1987), in order to improve purity of nucleic acids i.e. elimination of secondary metabolites, especially phenolics, the PVPP (insoluble and high molecular weight of cross-linked form of polyvinylpolypyrrolidone, Sigma-Aldrich Fine Chemicals) at concentrations ranged from 0 to 5% to the lysing buffer (GTC) was added.

RT-PCR reactions were performed as described previously (Cajza et al. 1997) and only thermal profile was slightly changed. After addition of templates and primers to the tubes preliminary denaturation for 5 min. was performed at 95°C. Then, after reduction of temperature to 42°C and addition of the rest of reaction components, reverse transcription was performed at 42°C for 50 min. followed by 5 cycles of amplification: 95°C, 1 min.; 50°C, 1 min.; 72°C, 1 min. and 25 amplification cycles: 95°C, 30 s; 55°C, 30 s; 72°C, 30 s. RT–PCR was performed in the Uno-Thermoblock thermocycler (Biometra).

RT-PCR reactions were performed employing primers specific for HLVd (pHLV1, 29-mer: 5' GAAGATCTCTGGGGAATACACTACGTGAC 3' and pHLV2, 29-mer: 5' GGGGTACCAGGGGCACTTTTTATGTGAAC 3') and HSVd (pHSVdU1, 27-mer: 5' CCGAGCTCCTGGGGAATTCTCGAGTTG 3' and pHSVdL2, 27-mer: 5' CCGGTACCAGGGGCTCAAGAGAGGATC 3').

With regard to restriction sites linked to the primers, lengths of products were 272 bp and 313 bp for HLVd and HSVd, respectively, as expected.

RT-PCR products were separated using 2% agarose gel. Electrophoresis was performed in the Agagel Mini apparatus (Biometra) using TBE buffer and ethidium bromide for staining of nucleic acids. Resulted patterns were visualized in UV light using a T13 transilluminator, Biometra.

RESULTS AND DISCUSSION

Significantly simplified method of Chomczynski and Sacchi (1987) appeared to be useful only for isolation of nucleic acids from hop plants grown in cultures *in vitro* giving templates of sufficient purity to perform RT-PCR tests for presence of viroids. In the case of material from hop plants and grapevine grown in natural conditions, purity of resulted nucleic acids was unsatisfactory for obtaining of repeatable results, regardless of type of organs tested and season of the year.

The simple way for improving of the purity of nucleic acids appeared to be addition of the insoluble form of PVPP to the GTC buffer. During homogenization of samples and further phenolic extraction insoluble PVPP binds secondary metabolites, especially phenolic derivatives. Therefore, after the first centrifugation PVPP with adsorbed metabolites sediments with the insoluble plant material and eliminates them from further steps of purification of nucleic acids giving clear supernatant of low viscosity.

Addition of PVPP to the lysing buffer did not complicate purification procedure and did not increase labor consumption.

Concentration of 2% PVPP in lysing buffer appeared to be very best for isolation of nucleic acids from hop plants as well as grapevine. The figure 1 illustrates results of RT-PCR tests for presence of HLVd in cultivable hop, where templates from leaves were purified using different concentrations of PVPP in the lysing buffer. Low concentrations, up to 1% PVPP appeared to be insufficient for obtaining templates of necessary purity. Using concentrations of PVPP higher than 3% products of RT-PCR were also not obtained. Nevertheless, it is worthy of note that method used for extraction of nucleic acids suitable for detection of HLVd in hop plants and HSVd in grapevines supported satisfactory results only till the end of June or first

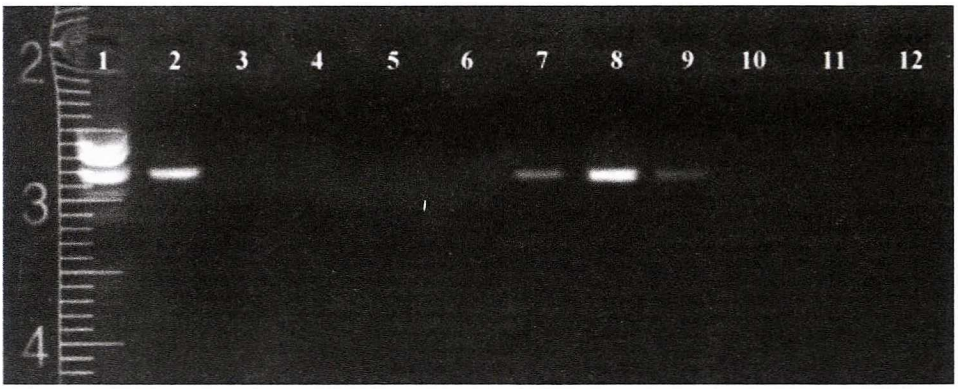

Fig. 1. Products of RT–PCR reaction of *Hop latent viroid* after extraction of nucleic acids using PVPP in the insoluble, high molecular weight form as an additive to the extraction buffer.

1. pUC18, *Hae* III;
2. Positive control – HLVd(+) – nucleic acids isolated from hop plants cv. Lubelski infected with HLVd and cultivated *in vitro* cultures;
3. Negative control – HLVd(–) – nucleic acids isolated from hop plants cv. Marynka HLVd free and cultivated *in vitro* cultures (2% PVPP in extraction buffer);
4. 0.0% PVPP in extraction buffer;
5. 0.5% PVPP in extraction buffer;
6. 1.0% PVPP in extraction buffer;
7. 1.5% PVPP in extraction buffer;
8. 2.0% PVPP in extraction buffer;
9. 2.5% PVPP in extraction buffer;
10. 3.0% PVPP in extraction buffer;
11. 4.0% PVPP in extraction buffer;
12. 5.0% PVPP in extraction buffer

Lanes 4–12 refer to the nucleic acids isolated from hop plants cv. Lubelski, infected with HLVd and cultivated in natural conditions

half of July. This correlates with growth stage of plants at a given vegetation season. In the case of hop plants it was to the end of flowering period. Tests performed later were characterized by decrease of repeatability, despite of higher concentration of viroids at that time than in spring and beginning of summer (Barbara et al. 1990; Morton et al. 1993).

In tests for presence of HLVd in hop plants in summer and fall presence of phenolic derivatives was not the factor disturbing RT-PCR reaction. Probably some other resin substances, characteristic for this plant species, which concentration systematically increased in this period were responsible for inhibiting RT-PCR.

High molecular weight, insoluble form of PVPP was used previously for purification of nucleic acids (Lodhi et al. 1994; Woodhead et al. 1997). However, according to other authors, better results could be obtained using high molecular weight PVP in soluble form (Maliyakal 1992) or low molecular weight, up to MW 40 kDa soluble one (Salzman et al. 1999), which is more effective and does not decrease the yield of purified nucleic acids.

It is known that soluble form of PVP (MW 20–30 kDa) can be use directly during the PCR test (Consul et al. 1999). However, this approach can be problematic

because PVP influences functioning of Taq polymerase so, it becomes difficult to determine the concentration of PVP in which unfavorable effect of phenolics would be eliminated and course of PCR would not be disturbed or blocked. According to the authors the very best results were obtained using 1% concentration of PVP however, in control reactions (without PVP and phenolics) more products of amplification were always obtained.

As a result of experiments performed and described in this paper, an effective and quick method for extraction of nucleic acids from hop plants and grapevine that were useful for detection of HLVd and HSVd by means of RT-PCR was elaborated. The optimal period for tests was spring and early summer when level of the secondary metabolites in plant tissues has been relatively low.

REFERENCES

Barbara D.J., Morton A., Adams N. 1990. Assessment of UK hops for the occurrence of hop latent and hop stunt viroids. Am. Appl. Biol., 116: 265–272.

Baxter N.J. 1997. Multiple interactions between polyphenols and salivary proline-rich protein repeat result in complexation and precipitation. Biochemistry 36: 5566–5577.

Cajza M., Wypijewski K., Pospieszny H. 1997. The use of reverse transcription and polymerase chain reaction (RT–PCR) for the detection of hop latent viroid (HLVd). J. Plant Protection Res., 37 (1/2): 52–58.

Chomczynski P., Sacchi N. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem., 162: 156–159.

Fang G., Hammar S., Grumet R. 1992. A quick and inexpensive method for removing polysaccharides from plant genomic DNA. Biofeedback 13 (1): 52–54.

Koonjul P.K., Brandt W.F., Farrant J.M., Lindsey G.G. 1999. Inclusion of polyvinylpyrrolidone in the polymerase chain reaction reverses the inhibitory effect of polyphenolic contamination of RNA. Nucl. Acids Res., 27: 915–916.

Levy L., Leel M., Hadidi A. 1994. Simple and rapid preparation of infected plant tissue extracts for PCR amplification of virus, viroid and MLO nucleic acids. J. Virol. Meth., 49: 295–304.

Lodhi M.A., Ye G.N., Weeden N.F., Reisch B.I. 1994. A simple and efficient method for DNA extractions from grapevine cultivars and *Vitis* species. Plant Mol. Biol. Rep., 12: 6–13.

Loomis W.D. 1974. Overcoming problems of phenolics and quinones in the isolation of plant enzymes and organelles. Methods Enzymol., 31: 528–545.

Maliyakal E.J. 1992. An efficient method for isolation of RNA and DNA from plants containing polyphenolics. Nucl. Acids Res., 20, p. 2381.

Morris J., Clover G.R., Harju V.A., Hugo S.A. 2001. Development of a highly sensitive nested RT–PCR method for beet necrotic yellow vein virus detection. J. Virol. Meth., 95: 163–169.

Morton A., Barbara D.J., Adams A.N. 1993. The distribution of hop latent viroid within plants of *Humulus lupulus* and attempts to obtain viroid-free plants. Am. Appl. Biol., 123: 47–53.

Peterson D.G., Boehm K.S., Stack S.M. 1997. Isolation of milligram quantities of nuclear DNA from tomato (*Lycopersicum esculentum*), a plant containing high levels of polyphenolic compounds. Plant. Mol. Biol. Rep., 15: 148–153.

Porebski S., Bailey L.G., Baum R. 1997. Modification of a CTAB DNA extraction protocol for plants containing high polysaccharide and phenol components. Plant Mol. Biol. Rep., 15: 8–15.

Salzman R.A., Fujikita T., Zhu-Salzman K., Hasegawa P.M., Bressan R.A. 1999. An improved RNA isolation method for plant tissues containing high level of phenolic compounds or carbohydrates. Plant Mol. Biol. Rep., 17: 11–17.

Soni R., Murray J.A. 1994. Isolation of intact DNA and RNA from plant tissues. Anal. Biochem., 218: 474–476.

Staub U., Polioka H., Gross H.J. 1995. Two rapid microscale procedures for isolation of total RNA from leaves rich in polyphenols and polysaccharides: application for sensitive detection of grapevine viroids. J. Virol. Meth., 52: 209–218.

Su X., Gibor A. 1988. A method for RNA isolation from marine macro-algae. Anal. Biochem., 74: 650–657.

Tesnierre C., Vayda M.E. 1991. Method for the isolation of high-quality RNA from grape berry tissues without contaminating tannins or carbohydrates. Plant Mol. Biol. Rep., 9: 242–251.

Wan C.Y., Wilkins T.A. 1994. A modified hot borate method significantly enhances the yield of high-quality RNA from cotton (*Gossipium hirsutum* L.). Anal. Biochem., 223: 7–12.

Wang H.M., Cutler A. 1993. A simple method for preparing plant samples for PCR. Nucl. Acids Res., 21: 4153–4154.

Woodhead M., Taylor M.A., Davies H.V., Brennan R.M., McNicol R.J. 1997. Isolation of RNA from blackcurrant (*Ribes nigrum*) fruit. Mol. Biotechnol., 7(1): 1–4.

Zhang Y.P., Uyemoto J.K., Kirkpatrick B.C. 1998. A small-scale procedure for extracting nucleic acids from woody plants infected with various phytopathogens for PCR assay. J. Virol. Meth., 71: 45–50.

POLISH SUMMARY

USPRAWNIONA METODA IZOLACJI CAŁKOWITYCH KWASÓW NUKLEINOWYCH Z CHMIELU I WINOROŚLI PRZED RT-PCR PRZEZ DODANIE POLIWINYLOPOLIPIROLIDONU

W przeprowadzonych doświadczeniach zastosowano nierozpuszczalną, wysokocząsteczkową formę PVPP do izolacji całkowitych kwasów nukleinowych z chmielu i winorośli w celu uzyskania matryc przydatnych do wykrywania HLVd i HSVd przy pomocy RT–PCR. Dodatek 2% PVPP do oryginalnego buforu ekstrakcyjnego GTC (Chomczynski i Sacchi, 1987) okazał się najkorzystniejszy. Dodatek PVPP umożliwił uproszczenie procedury ekstrakcji kwasów nukleinowych przez skrócenie czasu izolacji oraz obniżenie kosztów. Zastosowanie uproszczonej metody uzyskiwania matryc gwarantujących pełną powtarzalność wyników testów ograniczało się jednak do okresu wiosny i wczesnego lata.