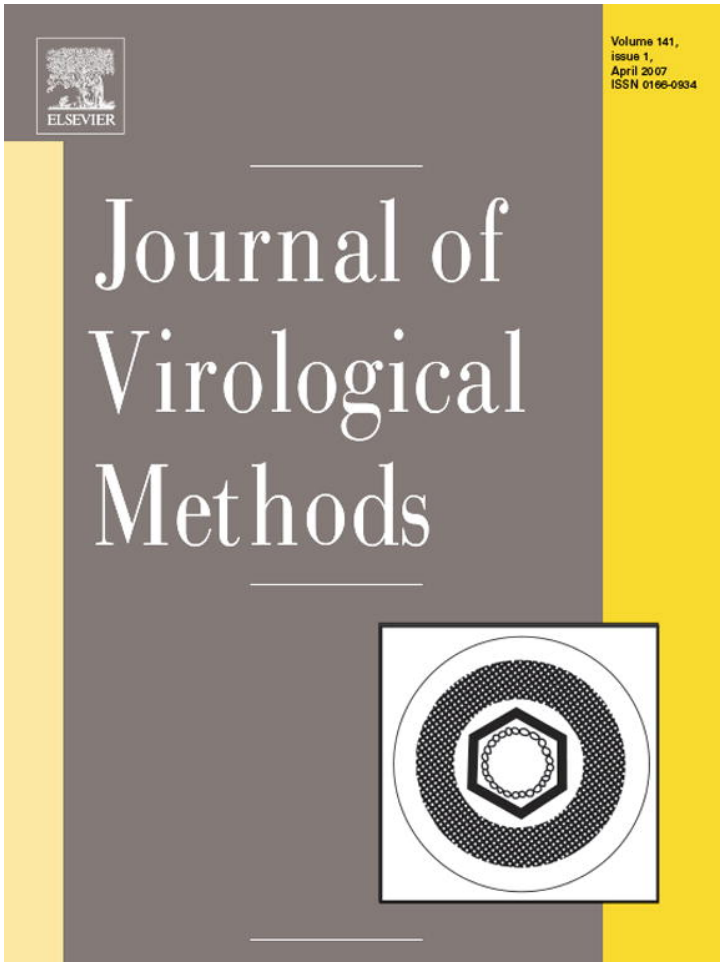

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Short communication

Sodium sulphite enhances RNA isolation and sensitivity of *Cucumber mosaic virus* detection by RT-PCR in black pepper

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Abstract

Isolation of intact high quality RNA suitable for RT-PCR from black pepper is greatly hindered by the presence of polyphenols and polysaccharides. These compounds adversely affect the sensitivity of virus detection by RT-PCR. The present study evaluated the effect of sodium sulphite in enhancing RNA yield and quality in a modified acid guanidium thiocyanate–phenol–chloroform (AGPC) protocol. The results were compared with the standard AGPC method and RNeasy Plant Mini Kit (Qiagen) for detection of *Cucumber mosaic virus* through RT-PCR. The addition of sodium sulphite in the extraction buffer increased the sensitivity of virus detection. Higher sensitivity of detection (than obtained from the kit) was seen when sodium sulphite was used at 0.5%. Similar levels of sensitivity were also observed for the detection of *Cucumber mosaic virus* from *Piper longum*.

Keywords: Black pepper; *Cucumber mosaic virus*; RT-PCR; RNA isolation; Sodium sulphite; Detection

Black pepper (*Piper nigrum* L.) is one of the most important spices in international commerce for many South East Asian countries including India. The productivity of the crop is reduced to a great extent due to adverse biotic and abiotic stresses. Among the biotic stresses, stunted disease caused by viruses is becoming increasingly important in all black pepper growing countries (de Silva et al., 2002). Association of a *Cucumber mosaic virus* has been established with stunted disease affected black pepper in India (Sarma et al., 2001). *Cucumber mosaic virus*, the type species of genus *Cucumovirus*, family *Bromoviridae* is one of the most widespread RNA virus of substantial agricultural importance, infecting more than 1000 plant species (van Regenmortel et al., 2000). The genome of the virus consists of plus-sense single stranded three RNAs (RNA 1, RNA 2 and RNA 3) and a subgenomic RNA (RNA 4) encoded by the 3′-half of RNA 3 (Palukaitis et al., 1992), which is involved in encapsidation (Suzuki et al., 1991). Numerous strains of *Cucumber mosaic virus* have been classified into two major subgroups (I and II) on the basis of serological properties and nucleotide sequence homology (Palukaitis et al., 1992). The subgroup I has been divided further into two groups (IA and IB) on the basis of gene sequences and phylogenetic analysis (Roossinck et al., 1999; Roossinck, 2002). Based on the coat protein sequence homology, CMV infecting black pepper was identified to be a strain of CMV belonging to subgroup IB (Bhat et al., 2005). External symptoms cannot be used as a sole criterion for confirming the disease free nature of the black pepper plant, since masking of symptoms is observed during certain seasons in most of the infected plants. Since black pepper is perennial in nature and propagated through vegetative means, it is necessary to develop a reliable and sensitive method for detection of the virus. Due to its high sensitivity, RT coupled with PCR in a single step is a molecular method used frequently for the detection of plant viruses (Bertolini et al., 2001).

The ability of isolating good quality RNA free of contaminating proteins, genomic DNA and secondary metabolites is crucial for RT-PCR. Isolation of nucleic acids from black pepper is greatly hindered by the presence of polyphenols and polysaccharides (Narayanan, 2000). These phenolic compounds increase the pigmentation of nucleic acids and inhibit adversely the detection of RNA targets in RT-PCR. The use of reducing agents in the extraction buffer is employed commonly to overcome the problem. Inclusion of sodium sulphite in the extraction buffer has been shown to prevent the degradation of DNA in *Acacia*

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(Byrne et al., 2001), improving the detection of viruses infecting cherry and potato in RT-PCR (Singh et al., 2002) and increasing the stability and quality of DNA in citrus (Baranwal et al., 2003). The present study evaluated the effect of sodium sulphite in increasing the efficiency of RNA isolation from black pepper by modifying the standard acid guanidium thiocyanate-phenol-chloroform (AGPC) method (Chomczynski and Sacchi, 1987) for sensitive detection of *Cucumber mosaic virus* in RT-PCR. The efficacy of this improved method was compared with that of a kit commercially available and standard AGPC method.

Infected black pepper isolates collected from different districts of Kerala and maintained in a glass house conditions at Indian Institute of Spices Research, Calicut, India were used in the present study.

Three different methods were used for RNA extraction. As a standard method, RNA was isolated using AGPC mixture (Chomczynski and Sacchi, 1987). In the second method, different concentrations of sodium sulphite (0.25%, 0.5%, 0.75%, 1%) were added to the extraction buffer as the final concentration and RNA was isolated as described below. Briefly, 50 mg of tender black pepper leaf were ground in 500 μl of denaturing solution (4 M guanidium thiocyanate, 25 mM sodium citrate pH 7.0, 0.5% (w/v) sodium *N*-lauryl sarcosine, 0.1% (v/v) β-mercaptoethanol). To this homogenate, 50 μl of 2 M sodium acetate pH 4.0, 500 μl of water–saturated phenol and 100 μl of chloroform: isoamyl alcohol (49:1) was added serially with intermittent mixing at each step. The tube was then shaken for 15 s and kept on ice for 15 min. The resulting homogenate was centrifuged at 12,000 × *g* for 15 min at 4 °C. To the aqueous phase, equal volume of isopropanol was added, incubated at −80 °C for 1 h and centrifuged at 12,000 × *g* for 15 min at 4 °C. The pellet was washed with 70% ethanol, air-dried and dissolved in 50 μl ultrapure water. For comparison, RNA was isolated from 50 mg of tender black pepper leaf using RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions and eluted in 50 μl elution buffer. Yield of RNA was determined by taking spectrophotometric readings. RNA extracted from healthy black pepper plants served as negative control. The experiments were repeated at least three times and the reproducibility checked.

RT and PCR were carried out in the same tube without any buffer changes in between. Primers designed from the coat protein gene of CMV of black pepper isolate (GenBank accession no: AY545924) were used to prime the reaction. The genome sense primer 5′-ATG GAC AAA TCT GAA TCA AC-3′ derived from the beginning of the first 20 bases of the coding region and the antisense primer 5′-TCA AAC TGG GAG CAC CC-3′ representing last 17 bases of the coding region of the CP gene were used to prime the reaction. A range of template volumes (10^{-4}, 10^{-3}, 10^{-2}, 10^{-1}, 1, 10 μl) was used in the RT-PCR. The RT-PCR was performed in a 50 μl reaction mixture containing 1× PCR reaction buffer (Genei, Bangalore, India), 10 mM DTT, 250 μM dNTPs (Merck, India), 15 pmol of each primers, 10 U of RNasin (NEB, Inc.), 50 U of MuMLV RT (Finnzymes, OY, Finland), 1.5 U Taq DNA polymerase (Genei, Bangalore, India). Samples were amplified in a thermocycler (Eppendorf Mastercycler Gradient) and the programme consisted of a reverse transcription step at 42 °C (45 min), followed by 40 cycle reaction profile involving denaturation of 94 °C (30 s), primer annealing at 50 °C (1 min), extension at 72 °C (1 min) with a final extension of 72 °C (10 min). The amplified products were analyzed on 1% agarose gel electrophoresis in TAE buffer, stained with ethidium bromide. The size of the amplicon was estimated by comparing with either a 100 or 500 bp DNA ladder (Genei, Bangalore, India). The bands were visualized and photographed using a transilluminator and gel documentation apparatus (Alpha Innotech Corporation, CA, USA).

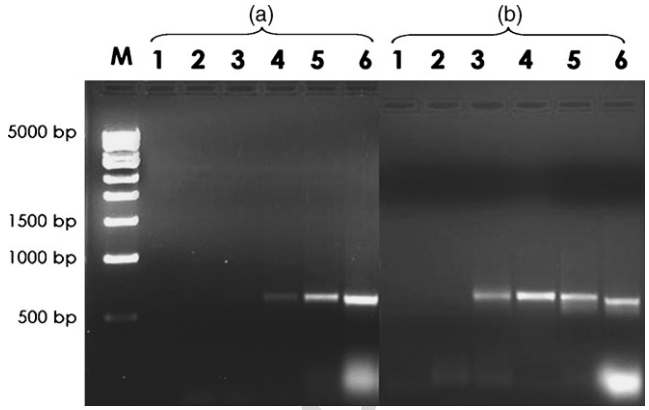

Fig. 1. Comparison of RT-PCR products derived from black pepper using standard AGPC method and Qiagen RNeasy Plant Mini Kit based isolation. (a) Standard AGPC method and (b) RNeasy Plant Mini Kit. Lane M: 500 bp DNA ladder; lanes 1–6: RNA at 10^{-4}, 10^{-3}, 10^{-2}, 10^{-1}, 1 and 10 μl, respectively.

RT-PCR amplified successfully the coat protein gene of *Cucumber mosaic virus* and a product of expected size (~650 bp) was observed in infected plants when RNA extracted from these three different methods was used as the template. No such band was seen in healthy samples. The identity of the amplicon was also confirmed by sequencing (data not shown).

In the initial studies, isolation of RNA was carried out using RNeasy Plant Mini Kit and standard AGPC method. When RNA isolated by these two methods was used for detection of *Cucumber mosaic virus* by RT-PCR, RNA isolated by kit was found to be more sensitive (Fig. 1a and b). But because of its cost, the standard AGPC method was modified to facilitate sensitive detection of the virus by inclusion of sodium sulphite in the extraction buffer.

When RNA extracted by these three methods was subjected to RT-PCR for the detection of *Cucumber mosaic virus* using different template volumes (ranging from 10^{-4} to 10 μl), the sensitivity of detection in the standard method ranged from 10^{-1} to 10 μl (Fig. 1a) while in the kit method, it was from 10^{-2} to 10 μl (Fig. 1b). In the modified method, using different concentrations of sodium sulphite, different levels of sensitivity were observed. Sensitivity was similar to that of the commercial kit when sodium sulphite was used at 0.25% (Figs. 1b and 2a) while at 0.75% it was similar to that of the standard AGPC method (Figs. 1a and 2c). The lowest sensitivity of detection was observed in 1.0% sodium sulphite (Fig. 2d). Highest level of detection (10^{-3} to 10 μl) was observed when sodium sulphite was used at 0.5% (Fig. 2b). When RNA extracted by all the three methods was compared, lowest yield of RNA was seen with the

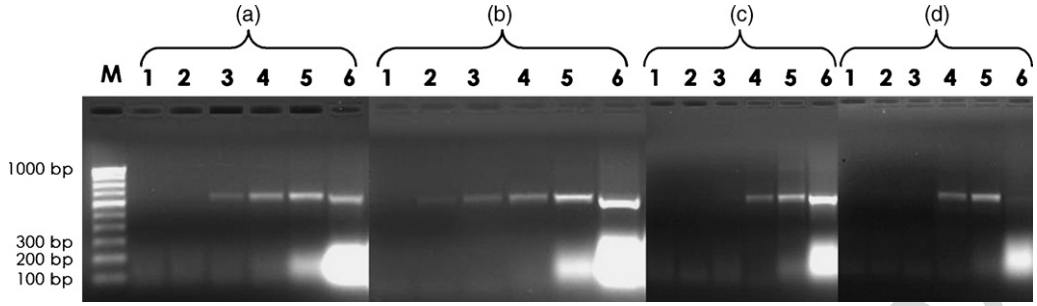

Fig. 2. Effect of sodium sulphite in extraction buffer on RT-PCR amplification at varying concentrations. (a) 0.25% Na_2SO_3; (b) 0.5% Na_2SO_3; (c) 0.75% Na_2SO_3; (d) 1.0% Na_2SO_3. Lane M: 100 bp DNA ladder; lanes 1–6: RNA at 10^{-4}, 10^{-3}, 10^{-2}, 10^{-1}, 1 and 10 μl, respectively.

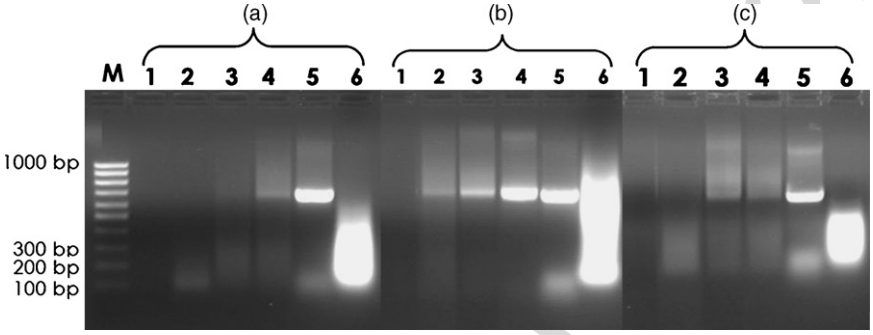

Fig. 3. Comparison of RT-PCR products from *Piper longum* using standard AGPC, RNeasy Plant Mini Kit and modified AGPC method with 0.5% sodium sulphite. (a) Standard AGPC method; (b) 0.5% sodium sulphite; (c) RNeasy Plant Mini Kit. Lane M: 100 bp DNA ladder; lanes 1–6: RNA at 10^{-4}, 10^{-3}, 10^{-2}, 10^{-1}, 1 and 10 μl, respectively.

standard AGPC method (112 ng/μl) followed modified method using sodium sulphite at 0.5% (536 ng/μl) and by commercial kit (670 ng/μl).

Thus, a concentration of sodium sulphite at 0.5% was found to be optimum for the amplification of the target RNA (Fig. 2b). This effective concentration of sodium sulphite established by the present study is comparable with that of 0.65–0.70% as cited by Singh et al. (2002) for the detection of potato and cherry viruses, and 0.65% as reported by Baranwal et al. (2003) for the detection of *Citrus yellow mosaic virus* in citrus leaves. The present study showed that increase in sodium sulphite concentration had an inhibitory effect on amplification. The precipitation of sulphite ions at high concentrations due to the acidic nature of the denaturing solution might have contributed to this inhibitory effect.

The addition of sodium sulphite in the buffer was a small modification but resulted in high sensitivity by detecting the virus at very low template volume. The same methodology was employed for isolating RNA from Indian long pepper (*Piper longum*) for the detection of *Cucumber mosaic virus*. The results of this study also confirmed that addition of 0.5% sodium sulphite enhanced the detection of *Cucumber mosaic virus* by RT-PCR when compared to the standard AGPC and a commercial kit based RNA isolation (Fig. 3). This increase in detection suggests that the addition of sodium sulphite provides good quality and stable RNA suitable for amplification. This might reveal the role of sodium sulphite as an effective reducing agent thereby preventing oxidation of nucleic acids in the plant sap as suggested by Byrne et al. (2001) and Baranwal et al. (2003). Since black pepper is propagated clonally by stem cuttings, the selection of virus free mother plants for further propagation is important. The simple cost effective RNA isolation method described in this paper can be used effectively for large scale indexing of viruses infecting black pepper and other *Piper* spp. through RT-PCR. Due to its high efficacy, the modified protocol may be suitable for the isolation of RNA which would facilitate sensitive detection of viruses from plants that are rich in polyphenols.

Acknowledgement

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