Antiviral Research 88 (2010) 296–303

Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

New in vitro method for evaluating antiviral activity of acyclic nucleoside phosphonates against plant viruses

J. Špak[a,*], A. Holý[b], D. Pavingerová[a], I. Votruba[b], V. Špaková[a], K. Petrzik[a]

[a] Biology Centre v.v.i., Institute of Plant Molecular Biology, Academy of Sciences, 37005 Banišovská 31, České Budějovice, Czech Republic
[b] Institute of Organic Chemistry and Biochemistry, v.v.i., Academy of Sciences, Flemingovo nám. 2, 166 10 Prague, Czech Republic

ARTICLE INFO

Article history:
Received 25 January 2010
Received in revised form
10 September 2010
Accepted 29 September 2010

Keywords:
Turnip yellow mosaic virus
Chemotherapy
Ribavirin
Brassica

ABSTRACT

A new method was developed for testing antiviral compounds against plant viruses based on rapidly growing brassicas in vitro on liquid medium. This method enables exchange of media containing tested chemicals in various concentrations and simultaneous evaluation of their phytotoxicity and antiviral activity. While using ribavirin as a standard for comparison, phytotoxicity and ability of the acyclic nucleotide analogues (*R*)-PMPA, PMEA, PMEDAP, and (*S*)-HPMPC to eliminate ssRNA *Turnip yellow mosaic virus* (TYMV) were evaluated by this method. Double antibody sandwich ELISA and real-time PCR were used for relative quantification of viral protein and nucleic acid in plants. Ribavirin had the most powerful antiviral effect against TYMV. On the other hand, (*R*)-PMPA and PMEA had no antiviral effect and almost no phytotoxicity compared to the control. (*S*)-HPMPC and PMEDAP showed moderate antiviral effect, accompanied by higher phytotoxicity. The tested compounds can be screened within 6–9 weeks in contrast to the 6 months for traditionally used explants on solid medium. The method enables large-scale screening of potential antivirals for in vitro elimination of viruses from vegetatively propagated crops and ornamentals.

© 2010 Elsevier B.V. All rights reserved.

1. Introduction

Plant virus chemotherapy has been defined as the production of virus-free plants from infected stock by chemical means. It is used for eliminating viruses from vegetatively propagated crops, such as fruits (Hansen and Lane, 1985; James et al., 1997), grapevine (Panattoni et al., 2007a,b), potato (Faccioli and Colombarini, 1996; Awan et al., 2007) and ornamentals (Lim et al., 1993; Verma et al., 2005), that are naturally infected by viruses. It is inevitably needed for eliminating complex infections of several viruses, as frequently occur in germplasm collections used in crop breeding and biodiversity programs (Spiegel et al., 1993).

Virus elimination biotechnology is often based on thermotherapy of whole plants or explants to reduce the virus concentration. This is followed by excising meristem tip tissue (cells not invaded by the virus) and its cultivation in vitro on media with an antiviral compound, frequently in a series of 3–5 passages. The success of virus elimination is verified by ELISA or PCR, repeated after months or even years (Verma et al., 2005), to assure the virus-free status of the plant material.

The possibility for phytotoxicity of antiviral compounds requires their testing in varying concentrations to establish an optimal balance between the virus elimination and percent of surviving explants.

It is evident from the literature that there are no ideal antiviral compounds and no ideal method for their evaluation. Lack of standardization in experimental methodologies for plant virus inhibition by chemicals can lead to contradictory conclusions. From a review of methods by Hansen (1989) (e.g. purified virus, virus-infected protoplasts, spraying virustatics on leaves or even on whole plants, floating leaf disk method, callus treatment, meristem treatment, injection into a whole plant or drench application to roots), there seems to be a general tendency toward increasing specificity of inhibitory response with increasing degree of plant tissue organization. Meristem treatment with antivirals, which reduces or completely prevents virus replication in growing parts, has excellent predictability for therapeutic success. This is a very time-consuming method, however, and it is not optimal for testing numerous antiviral compounds.

Testing of newly synthesized compounds or those found effective in human or animal medicine could bring advances in plant virus elimination, although their effect against plant viruses may be different. Among possibly effective compounds

Abbreviations: ANP, acyclic nucleoside phosphonate; PMEA, 9-[2-(phosphonomethoxy)ethyl]adenine; PMEDAP, 2,6-diamino-9-[2-(phosphonomethoxy)ethyl]purine; (*R*)-PMPA, (*R*)-9-[2-(phosphonomethoxy)propyl]adenine; (*S*)-HPMPC, (*S*)-1-[3-hydroxy-2-(phosphonomethoxy)propyl]cytosine; DAP, 2,6-diaminopurine; DAPy, 2,6-diaminopyrimidine.

* Corresponding author. Tel.: +420 385 310 357; fax: +420 385 310 356.
E-mail address: spak@umbr.cas.cz (J. Špak).

0166-3542/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.antiviral.2010.09.019

might be acyclic nucleoside analogues (prototype (*S*)-DHPA) and a large number of nucleotide analogues (acyclic nucleoside phosphonates (ANPs), prototype (*S*)-HPMPA). Many other ANPs, such as 2,6-diaminopurine (DAP) and 2,6-diaminopyrimidine (DAPy) derivatives, have been found to exhibit antiviral activity spectra and potency comparable to those of the parent compounds (*S*)-HPMPA and (*S*)-HPMPC, PMEA and (*R*)-PMPA, respectively (De Clercq, 2007). After the intracellular phosphorylation the diphosphates of PMEA and PMEDAP (i.e. PMEApp and PMEDAPpp), analogues of nucleoside 5′-triphosphates, inhibit both viral DNA polymerases (Kramata et al., 1996; Birkuš et al., 1999) and reverse transcriptases (Naesens et al., 1997). Accordingly, (*R*)-PMPApp is a potent inhibitor of the reverse transcriptase activity (Suo and Johnson, 1998) but not of DNA polymerases (Birkuš et al., 2002). None of these compounds have thus far been studied for their possible abilities to inhibit RNA polymerase activity of plant viruses. Contrary to purine ANPs, which terminate DNA growing chain *de novo*, (*S*)-HPMPCpp is incorporated into DNA nascent chain up to three residues (Birkuš et al., 2001).

The aim of this study was to develop a simple method suitable for low-cost and rapid screening of numerous ANPs for their antiviral activity. Moreover, we first tested phytotoxicity and ability of the acyclic nucleotide analogues (*R*)-PMPA, PMEA, PMEDAP and (*S*)-HPMPC to eliminate ssRNA *Turnip yellow mosaic virus* (TYMV) in comparison to ribavirin, which was used as a standard. According to the literature (Hansen, 1989), ribavirin seems to yield the best antiviral effect against RNA plant viruses. TYMV and fast-growing *Brassica pekinensis* were selected as low-cost and suitable model organisms to validate the method.

2. Materials and methods

2.1. Antiviral compounds

Ribavirin (1-(β-D-ribofuranosyl)-1H-1,2,4-triazole-3-carboxamide) was purchased from Duchefa Biochemie B.V. (Haarlem, the Netherlands). The ANPs (*R*)-PMPA, PMEA, (*S*)-HPMPC and PMEDAP were synthesized as previously described by Holý et al. (1989) These include compounds that have been approved worldwide for clinical use:

(1) (*S*)-HPMPC {1-[(*S*)-3-hydroxy-2-(phosphonomethoxy)propyl] cytosine, cidofovir, Vistide®} for the treatment of cytomegalovirus retinitis in AIDS patients and treatment of polyoma-, papilloma-, adeno-, herpes- and poxvirus infections (dsDNA genome);
(2) PMEA {9-[2-(phosphonomethoxy)ethyl]adenine, adefovir} for the treatment of chronic hepatitis B virus infections (dsDNA genome), and
(3) (*R*)-PMPA {9-[(*R*)-2-(phosphonomethoxy)propyl]adenine, tenofovir} for the treatment of HIV (retrovirus) infection (De Clercq, 2007).

2.2. In vitro cultivation of plants

Seeds of *B. pekinensis* (Lour.) Rupr., cv. Manoko (30 in a 2 ml Eppendorf tube) were surface-sterilized by immersion in 10% commercial bleach (SAVO, Bochemie, Czech Republic, approximately 3% sodium hypochlorite) for 10 min and washed 5 times with sterile water. The seeds were transferred aseptically to polycarbonate Magenta® vessels for plant tissue culture (Sigma–Aldrich, height 97 mm) (Fig. 1) containing filter paper bridges and 12 ml of Murashige and Skoog's (MS) liquid medium including vitamins (2.0 mg/l glycine, 100 mg/l myo-inositol, 0.5 mg/l nicotinic acid, 0.5 mg/l pyridoxine and 0.1 mg/l thiamine) and 20 g/l sucrose

Fig. 1. *Brassica pekinensis* cv. Manoko plant cultivated in magenta on a paper bridge soaked with cultivation medium.

(Murashige and Skoog, 1962). The plants were cultivated throughout the experiment under a 16 h photoperiod, at a temperature of 23 °C and light intensity of 90 μmol/m^{-2}/s.

2.3. Aseptic virus inoculation

Plants grown in Magenta® vessels were ready for mechanical inoculation with the virus within 2 weeks. Czech isolate of *Turnip yellow mosaic virus* (TYMV) (Polák and Špak, 1987) maintained in the virus collection at the Institute of Plant Molecular Biology was used for this study. Singular virus infection was checked by sucrose density gradient during the virus purification, electron microscopy, preparation of antiserum and serology. The inoculum was prepared from infected *B. pekinensis* cv. Manoko plants with mosaic symptoms 3 weeks after inoculation. A leaf sample was homogenized in a mortar in a 1:10 (w/v) ratio with 0.1 M phosphate buffer, pH 7.0, and filtered through 0.22 μm sterile filters (Millipore). Mechanical inoculation of plants was conducted on the first 2 leaves in a flowbox using a cotton pad soaked in inoculum mixed with carborundum powder. Control plants were inoculated only with buffer.

2.4. Experimental scheme for using ELISA to assess virus inhibition

(a) Germination of seed in Magenta® vessels on liquid medium.
(b) Mechanical inoculation of 250 plants (2 weeks old) with the virus.
(c) Week 5: Selection of infected plants and application of antiviral compounds. The first ELISA was conducted 3 weeks after plants were inoculated with virus. Infected plants with absorbances at 405 nm higher than the mean ± 3 S.D. (Sutula et al., 1986) of noninfected control plants were selected for the testing of antiviral compounds. Groups of infected plants with minimum difference in mean absorbance were established for each compound and control. Groups of 16 and 17 plants for each

compound, both infected and uninfected control, were used in the first and second test, respectively. Acyclic nucleotide analogues (*R*)-PMPA, PMEA, PMEDAP, (*S*)-HPMPC and ribavirin (Duchefa) were dissolved in liquid MS medium to the concentration of 50 mg/l and filtered through 0.22 μm sterile filters (Millipore). Application of antiviral compounds was carried out by replacing medium in Magenta® vessels by pipetting immediately after the first ELISA.

(d) Weeks 8, 11, 14 and 17: Estimation of virus concentration by ELISA, exchange of medium, real-time PCR test, and observation of phytotoxic effect. Leaf samples for subsequent ELISA were taken from plants in 3-week intervals. Six ml of fresh media containing the antiviral compounds was added to the Magenta® vessels after each sampling. Real-time PCR test of plants with the lowest absorbance values in ELISA was conducted. Numbers of vital, yellowing and dying out plants were recorded. The experiment was repeated twice and the results are reported as test 1 and test 2.

2.5. ELISA

Virus inhibition expressed as relative virus concentration in plants was estimated by standard double antibody sandwich ELISA (Clark and Adams, 1977) using TYMV antibodies conjugated with alkaline phosphatase. Antibodies are commercially available through Bioreba AG, Switzerland. Details of buffers used are available at http://www.bioreba.ch/. Ninety-six well plates were coated with immunoglobulin G at a concentration of 2 μg/ml and incubated for 4 h at 36 °C. Test samples were taken from plants in a flowbox. All but the first true leaf were excised, weighed and homogenized in Bioreba extraction bags with standard Bioreba homogenization buffer in a ratio of 1:10 using the Homex device (Bioreba, Switzerland). After washing the plates, the samples were pipetted and incubated overnight in a refrigerator at 4 °C. Each plant sample was pipetted twice. After washing, the conjugate was pipetted and incubated for 4 h at 36 °C. Then 4-nitrophenol substrate was added and after 40 min the absorbance at 405 nm was measured in a Tecan Spectra Classic ELISA reader and evaluated using KIM-W software. Calibration of ELISA was based on data obtained for purified virus in preliminary experiments (data not shown). Based on this data ELISA (IgG for coating, dilution of plant sap, dilution of conjugate and incubation time) was set up to be linear within the absorbance range of the tested plant samples. Identical positive and negative control samples were used for comparing the results in individual plates throughout the experiment.

The ELISA absorbance data were evaluated by one-way ANOVA followed by Tukey's Honestly Significant Differences Test (HSD test) using Statistica (StatSoft Inc.) software.

2.6. Real-time polymerase chain reaction (PCR)

Infected plants with the lowest absorbance values in ELISA at 6, 9 and 12 weeks after application of potential antivirals were selected for more sensitive evaluation of virus concentration using real-time PCR. The RNA for reverse transcription and real-time PCR was isolated from about 0.1 g of infected leaves using a Nucleospin RNA plant kit (Macherey Nagel, Germany) according to the manufacturer's recommendations and eluted with 30 μl of water. Complementary DNA was synthesized from 7 μl of RNA using an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) in 10 μl reaction volume. TYMV-specific primers were used to amplify a 198 bp segment of the capsid protein gene (Petrzik, 2009). An internal transcribed spacer of the plant ribosomal gene about 300 nt long and amplified with primers 5′-CGTTGAGGACTTTGGGTCAT-3′ and 5′-GCGAAATGCGATACTTGGTG-3′ was used as internal control in a parallel reaction. The 20 μl PCR reaction mixture contained 10 mM Tris–HCl, pH 8.8, 50 mM KCl, 0.1% Triton X-100, 1.5 mM $MgCl_2$, 200 μM each of dNTPs, 1 U Taq DNA polymerase, 2 μl of cDNA, 20 pmol of TYMV or ribosomal primers, and SYBR green stain. The amplifications were performed in an iCycler (Bio-Rad) with an amplification program of 40 cycles with 20 s denaturation at 95 °C, 20 s annealing at 58 °C, and 20 s synthesis at 72 °C. All samples were analyzed using 1.5% agarose gel electrophoresis.

Samples were rated as negative if no band of expected size (198 bp) was visible in samples with threshold value (C_T) above 25. The virus concentration relative to the assumed 100% amplification efficiency was calculated as $2^{C_T\ PC47 - C_T\ sample}$, where C_T PC47 was a positive control not treated by ribavirin.

2.7. Assessing the phytotoxicity of compounds

PMEA, which was available in a sufficient amount, was selected for preliminary testing to set up an appropriate concentration of ANPs. PMEA was dissolved in liquid MS medium to the concentrations 25, 50, 75 and 100 mg/l and added to 3-week-old plants in Magenta® vessels by exchange of the pure MS medium. Five plants were used for each concentration and pure MS medium was used as control. After 4 weeks, the fresh weight of hypocotyl and leaves was recorded.

Based on results from preliminary testing with PMEA, the concentration of 50 mg/l was selected for phytotoxicity testing of all compounds. Uninfected plants of *B. pekinensis* were cultivated in Magenta® vessels as described in Section 2.2. Acyclic nucleotide analogues (*R*)-PMPA, PMEA, PMEDAP, (*S*)-HPMPC and ribavirin (Duchefa) were dissolved in liquid MS medium to the concentration of 50 mg/l, filtered through 0.22 μm sterile filters (Millipore) and added to 3-week-old plants in Magenta® vessels through exchange of the pure MS medium by pipetting. Thirty plants each were used for ribavirin, PMEA and control (pure MS medium); 12 plants each were used for PMEDAP, (*S*)-HPMPC and (*R*)-PMPA. After 6-week cultivation, the dry weight (drying for 24 h at 90 °C) of hypocotyl and leaves was recorded. Data were evaluated by ANOVA followed by Tukey's HSD test using Statistica (StatSoft Inc.) software.

2.8. Determination of ANPs and ribavirin levels in the cultivation media

Freshly prepared MS media (control and containing 50 mg/l of ANPs and ribavirin) and 1 ml samples from 5 randomly selected Magenta® vessels (control and each compound) were taken at the beginning of the experiment and after 3-week cultivation of TYMV-infected plants (test 1 and test 2) and stored at −20 °C until the analysis. The diluted aliquots of media were analyzed in the Waters Alliance HPLC system (996 PDA Detector, PDA Empower Pro Software, version 2) equipped with a 15 cm × 4.6 mm SUPELCOSIL™ LC 18T, 3 μm reverse-phase column. A nonlinear gradient of acetonitrile (0–21%) in 50 mM potassium phosphate, pH 3.1, 3 mM tetrabutylammonium bisulfate at a flow rate of 0.75 ml/min was used for media containing PMEA, PMEDAP and (*R*)-PMPA. (*S*)-HPMPC and ribavirin were analyzed at pH 5.2 in the same solvent system. Before the injection, the samples were diluted with HPLC water in a 1:3 ratio (0.1 ml of sample and 0.3 ml of water) and filtered through a 0.2 μm filter. Peaks of the corresponding compounds were identified using UV-spectra library and quantified with the aid of external standards. The described method enables the separation of original compounds and their phosphorylated derivatives. The analysis was repeated twice, and in the case of ribavirin 3 times.

Determination of ANPs and ribavirin levels in the cultivation media in the phytotoxicity experiment (Section 2.6) with uninfected plants was conducted using the same procedure. Freshly prepared MS media (control and containing 50 mg/l of ANPs and

Table 1
Evaluation of phytotoxicity of ribavirin and acyclic nucleoside phosphonates (*S*)-HPMPC, PMEDAP, (*R*)-PMPA and PMEA in concentration 50 mg/l. Values represent % of surviving *Brassica pekinensis* cv. Manoko plants infected with *Turnip yellow mosaic virus* during the experiment. Summarized data from test 1 and test 2.

Compound	Weeks				
	0	3	6	9	12
Ribavirin	100.0	96.7	80.0	76.7	66.7
(*S*)-HPMPC	100.0	96.7	86.7	63.3	3.3
PMEDAP	100.0	96.7	96.7	63.3	6.7
(*R*)-PMPA	100.0	100.0	83.3	70.0	53.3
PMEA	100.0	100.0	100.0	96.7	73.3
Infected control	100.0	93.3	83.3	76.7	73.3
Uninfected control	100.0	100.0	90.0	90.0	90.0

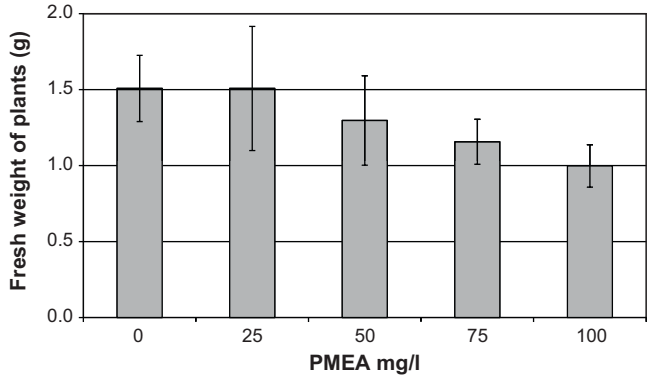

Fig. 2. Phytotoxicity test: fresh weight of uninfected *Brassica pekinensis* cv. Manoko plants after 4-week cultivation on Murashige and Skoog's liquid medium containing different PMEA concentrations.

ribavirin) and 1 ml samples from 3 randomly selected Magenta® vessels of each compound were taken at the beginning of the experiment and after 6-week cultivation of uninfected plants and stored at −20 °C until the analysis. MS medium without plants was used as control.

3. Results

3.1. Assessment of phytotoxicity

PMEA, which was available in a sufficient amount for the experiments, was selected to set up an appropriate concentration of ANPs for the experiments with *B. pekinensis*. The results are summarized in Fig. 2. Statistical analysis revealed no significant difference in fresh weight of plants at $P = 0.05$. However, the P value (0.091) for the 100 mg/l concentration was close to the significance limit compared to the control. The phytotoxicity experiment conducted with all compounds at the concentration 50 mg/l (Fig. 3) showed decrease in dry weight of plants treated with (*S*)-HPMPC and PMEDAP. Only dry weight of (*S*)-HPMPC treated plants was significantly lower ($P < 0.05$) than those treated by other compounds and control. Yellowing of older leaves was observed with these compounds, but all plants survived until the end of the experiment. Reduction of dry weight was statistically significant only in plants treated with (*S*)-HPMPC. Finally, the concentration 50 mg/l was selected for all ANPs and ribavirin as sufficient to reveal both the potential antiviral and phytotoxic effects. The data from the phytotoxicity experiment (Fig. 3) correlated well with the survival of plants during the assessment of virus inhibition by ELISA in TYMV-infected plants summarized in Table 1. Differences in phytotoxicity of the compounds were visible from the 6th week after application of the compounds. Ribavirin, (*R*)-PMPA and PMEA had almost no phytotoxicity compared to the control after 9 weeks of cultivation. (*S*)-HPMPC and PMEDAP revealed moderate phytotoxicity, visible as yellowing of plants from the 6th week and dramatic dying out between the 9th and 12th weeks.

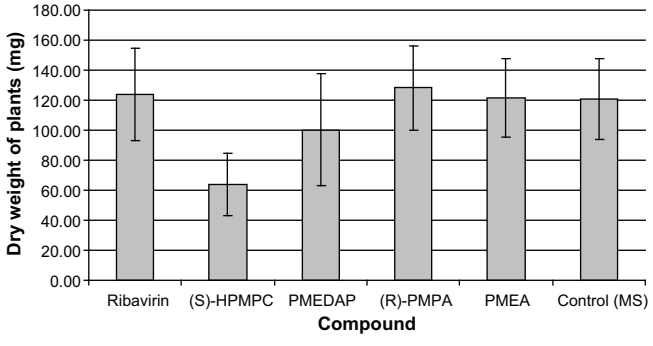

Fig. 3. Phytotoxicity test: dry weight of uninfected *B. pekinensis* cv. Manoko plants after 6-week cultivation on liquid MS medium containing ribavirin and acyclic nucleoside phosphonates (*S*)-HPMPC, PMEDAP, (*R*)-PMPA and PMEA in concentration 50 mg/l.

3.2. Assessment of virus inhibition

The data are summarized in Table 2 and Fig. 4a and b. (*R*)-PMPA and PMEA had no antiviral effect, while (*S*)-HPMPC and PMEDAP

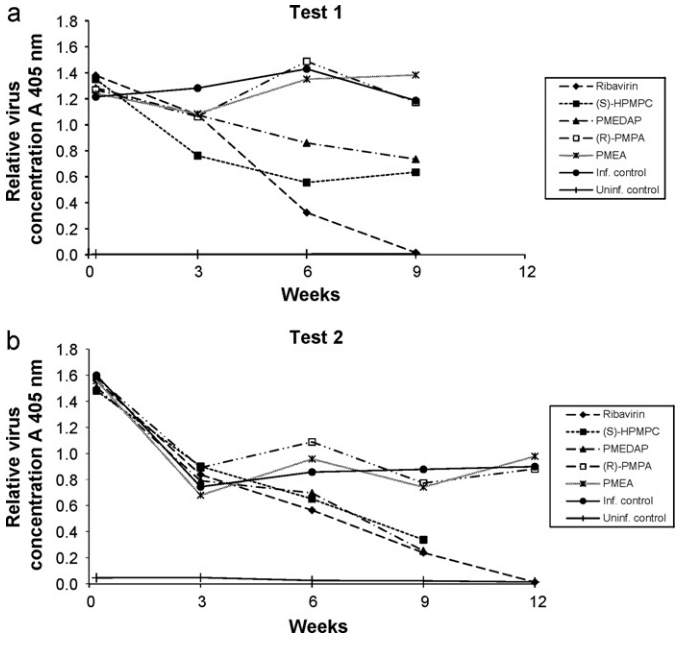

Fig. 4. The effect of ribavirin and acyclic nucleoside phosphonates (*S*)-HPMPC, PMEDAP, (*R*)-PMPA and PMEA at concentration 50 mg/l on *Turnip yellow mosaic virus* relative concentration in *Brassica pekinensis* cv. Manoko plants. Relative virus concentration is expressed as absorbance at 405 nm in ELISA.

Table 2
Statistical evaluation of relative *Turnip yellow mosaic virus* concentration in *Brassica pekinensis* cv. Manoko plants. Plants were cultivated 9 weeks on MS liquid medium supplemented with ribavirin and acyclic nucleoside phosphonates (*S*)-HPMPC, PMEDAP, (*R*)-PMPA and PMEA in concentration 50 mg/l. Values represent mean and S.D. absorbance at 405 nm in ELISA and results of Tukey's Honestly Significant Differences Test (HSD test).

Compound	Test 1			Test 2		
	A 405 nm			*A* 405 nm		
	Mean	S.D.	Tukey's HSD test	Mean	S.D.	Tukey's HSD test
Ribavirin	0.016	0.01	$P < 0.05$	0.238	0.39	$P < 0.05$
(*S*)-HPMPC	0.635	0.27	$P < 0.05$	0.338	0.17	$P < 0.05$
PMEDAP	0.735	0.26	$P < 0.05$	0.253	0.15	$P < 0.05$
(*R*)-PMPA	1.383	0.51	NS	0.742	0.31	NS
PMEA	1.173	0.37	NS	0.772	0.34	NS
Infected control	1.186	0.42	NS	0.878	0.28	NS
Uninfected control	0.023	0.03	$P < 0.05$	0.008	0.02	$P < 0.05$

revealed similar moderate antiviral effect in comparison to the control, with significant difference at $P < 0.05$ in the 9th week. Ribavirin revealed the best antiviral effect. The second test was started with higher virus concentration in plants, which led to a delay in decrease of virus content. The reason for higher virus concentration could be natural variation in virus replication after inoculation of plants. In contrast to the first test, there was no significant difference between ribavirin and (*S*)-HPMPC and PMEDAP in the second test in the 9th week. This difference resulted from higher S.D. of the ribavirin-treated plants, as two plants remained with higher virus content as of the 9th week.

Analysis of relative virus content in individual plants revealed a sudden drop in TYMV concentration in most plants treated with ribavirin, although in some of them the virus persisted for longer periods. This depended on the initial virus concentration. In the first test, already after 6 weeks of treatment 7 of 12 plants had absorbance values close to the ELISA detection limit (Table 2). In the 9th week, no plant was positive by ELISA. A similar situation occurred in the second test, where 6 and 8 plants were negative by ELISA in the 6th and 9th weeks, respectively, and only 1 of 10 surviving plants was positive at the end of the experiment. The correlation coefficients between the relative virus concentration and time (0–9 weeks) in test 1 were −0.753, −0.534 and −0.421 for ribavirin, (*S*)-HPMPC and PMEDAP, respectively.

In contrast to ribavirin treatment, the decrease of virus content in plants treated with (*S*)-HPMPC and PMEDAP was slower. The virus remained detectable by ELISA in the 9th week for both experiments. Exceptional were two PMEDAP-treated plants in the second test. The data indicated that the reduced virus content resulted from virus inhibition by the compounds rather than from their phytotoxicity. Significant decrease of relative virus content was visible already in the 6th week in both experiments when plants showed no yellowing or growth reduction (Fig. 4a and b). Also, surviving plants in both PMEDAP and (*S*)-HPMPC groups in the 9th week was only 6.7% lower (Table 1) than for (*R*)-PMPA-treated plants, which showed no virustatic effect.

Real-time PCR analysis of plants that were negative by ELISA in the 6th week (test 1) is summarized in Table 3. The threshold value (C_T) of internal controls was 9.7 ± 0.4 within the 95% confidence interval. The C_T of TYMV-evaluated plants ranged from 10 to 22. The drop of viral dose ranged from 1:630 to 1:2194 in comparison to the positive control (C 47) not treated by ribavirin. Plants 72 and 110 did not give specific amplification products and their C_T values were not used in the calculations. Real-time PCR results in Table 3 revealed differences in the relative amount of viral nucleic acid in ELISA-negative plants treated with ribavirin that were not visible by agarose gel electrophoresis (Fig. 5a). Fig. 5a and b displays the differences in PCR product obtained in the 6th and 9th weeks, respectively. While only two plants were negative (no visible bands) in the 6th week, samples taken 9 weeks after ribavirin treatment gave no or not specific products.

3.3. Determination of ANPs and ribavirin levels in the cultivation media

The data from systematic medium analysis after the dilution of samples in a 1:3 ratio show that the medium components do not interfere with individual ANPs or with their putative metabolites. The results from HPLC analysis of ribavirin and ANPs content in medium after 3-week cultivation of TYMV-infected plants and 6-week cultivation of uninfected plants and control without plants are summarized in Table 4. The numbers represent percent of initial concentration of compounds at the beginning of experiments. The data of control samples indicate that the compounds are stable in the medium even after 6 weeks. The increase of concentration was due to the evaporation of medium. The concentrations of ANPs and

Table 3
Relative quantitative values of *Turnip yellow mosaic virus* protein and RNA of *Brassica pekinensis* cv. Manoko selected plants 6 weeks after treatment with ribavirin. Relative virus protein content is expressed as absorbance values at 405 nm in ELISA, relative RNA content is expressed as relative amount of virus against nontreated positive control PC 47 after amplification by real-time PCR (test 1).

Plant no.	ELISA *A* 405		Real-time PCR	
	Mean	S.D.	Threshold value C_T	Relative amount of virus against PC 47
48	0.031	0.008	21.3	1/1448
64	0.087	0.004	20.1	1/630
72	0.010	0.008	38.5	–[a]
90	0.018	0.000	21.3	1/1448
102	0.020	0.004	21.9	1/2194
104	0.014	0.001	20.4	1/776
110	0.004	0.002	28.5	–[a]
PC 47	1.696	0.110	10.8	1

PC 47, positive control not treated with ribavirin.
[a] Plants 72 and 110 yielded no specific amplification products, and therefore their C_T values were not used for calculation.

Table 4
HPLC analysis of ribavirin and acyclic nucleoside phosphonates content in MS liquid medium after cultivation of *Brassica pekinensis* cv. Manoko plants infected with *Turnip yellow mosaic virus* for 3 weeks, uninfected plants and control MS medium (no plants) for 6 weeks. Values represent mean % and standard deviation (S.D.) of initial concentration of compounds (50 mg/l) at the beginning of experiments.

Compound	TYMV infected plants		Uninfected plants		Control
	Mean	S.D.	Mean	S.D.	Mean
Ribavirin	86.9	13.9	63.1	2.5	107.2
(*S*)-HPMPC	88.0	9.7	90.2	6.9	139.0
PMEDAP	54.7	12.7	58.2	26.4	115.6
PMEA	66.8	16.0	95.0	12.0	102.8
(*R*)-PMPA	99.7	23.2	90.2	8.3	112.9

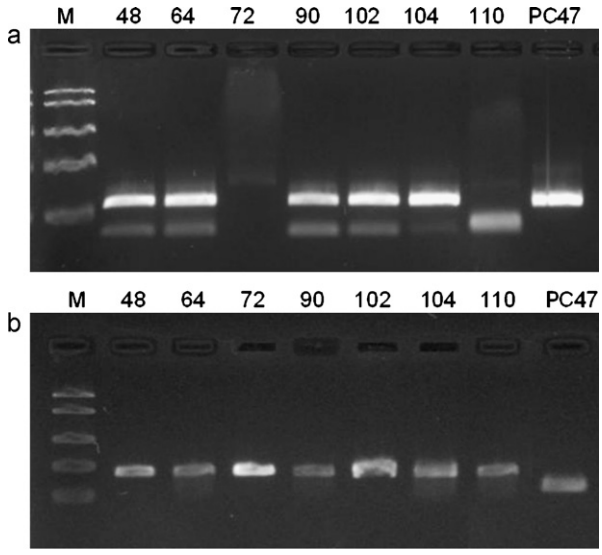

Fig. 5. Agarose gel analysis of products after 40 cycles of real-time PCR amplification of *Turnip yellow mosaic virus* RNA in test 1: (a) Selected ELISA negative plants 6 weeks after ribavirin treatment. All but plants 72 and 110 gave band of expected size. M – marker, PC47 – untreated positive control. (b) Selected ELISA negative plants 9 weeks after ribavirin treatment. Samples gave no or not specific products.

ribavirin were decreasing both in TYMV-infected and -uninfected plants. It should be noted that we identified no metabolites of the title compounds in the media at a measurable concentration.

4. Discussion

The data from both tests demonstrated that already during 6–9 weeks post-application it is possible to select compounds with antiviral effect for further tests. This discrimination is of particular importance for the preselection of numerous derivatives of potential antivirals prepared by chemists.

Rapid growth of brassicas, easy preparation of plants for the tests, and shortening of test time from the 6 months in meristem/solid agar testing (Helliot et al., 2003) to 6 weeks are additional advantages. However, with some well-regenerating plant species, e.g. potato (Faccioli and Colombarini, 1996), orchid (Lim et al., 1993) and apple (Hansen and Lane, 1985), the test time was comparable to that for meristem/solid agar testing. *B. pekinensis* plants grown in Magenta® vessels are ready for virus inoculation within 2 weeks and ready for the tests 3 weeks after infection. With potato or tobacco, the plants most frequently used in published experiments (e.g. Stahl and Sander, 1992), the preparation of plant material requires several months. Similarly, raising and multiplying explants from virus-infected plants is very time-consuming (Lerch, 1987; Panattoni et al., 2007a). To derive virus-infected in vitro shoot cultures from e.g. small field fruits and fruit trees (non-herbaceous species) in sufficient numbers for the tests takes months or even a year in contrast to the 5 weeks necessary for model plants in Magenta® vessels. Numerous RNA and DNA viruses infecting brassicas can be selected for the tests. The method has potential application to other plant taxa and plant–virus-compound combinations, including the possibility for reducing the virus content in seedlings before excision of meristems.

Cultivating plants in Magenta® vessels on a paper bridge in liquid medium enables easy addition of antivirals in varying concentrations and excludes the necessity of more laborious passage on semi-solid MS medium. In contrast to meristem treatment on solid agar, it brings an additional advantage in the possibility for monitoring the virus content in individual plants for a long period. Moreover, it rapidly shows the phytotoxicity, which can be evaluated by change of color and reduced growth of plants. In vitro cultivation in air-conditioned rooms ensures standard light and temperature conditions throughout an entire experiment.

The validation of the new method by ribavirin yielded results comparable to those obtained by other methods (Lerch, 1987). ELISA is less expensive than PCR, and it is sufficient for estimating virus concentration. In order to eliminate the possible false positive reaction with empty protein shells in ELISA (Francki and Matthews, 1962) and to verify the complete virus elimination, we used a more sensitive real-time PCR. This confirmed the data obtained by ELISA.

Ribavirin revealed a better virustatic effect against ssRNA *Turnip yellow mosaic virus* than did the ANPs. Our experiments confirmed the necessity for the plants to have long-term exposure to the compounds in order to reduce the virus content. Ribavirin has been tested under a wide range of experimental conditions and has been found to be effective against at least 20 plant viruses (Hansen, 1989). Its metabolism and antiviral activity were reviewed by Parker (2005), and its antiviral mechanism in plants was studied by Quecini et al. (2006). It is used in many laboratories to produce virus-free plants (e.g. Verma et al., 2005; Awan et al., 2007). Virus-free shoots can be produced on meristems by incorporating 5–50 mg/l of ribavirin into the medium. Ribavirin seems to yield the best results against RNA plant viruses, which represent approximately 75% of all known viruses invading plants (Büchen-Osmond, 2006; http://www.ictvdb.org/). Therefore, we involved ribavirin in our study not only for the sake of validating the new method but also as a standard for evaluating novel antiviral candidates combating plant viruses (Xia et al., 2006; Panattoni et al., 2007b). Promising, newly tested antivirals should be superior to ribavirin in their antiviral potency, phytotoxicity and costs.

To the best of our knowledge, none of the ANPs had previously been tested for inhibiting RNA polymerase activity of plant RNA viruses. TYMV, a positive-strand RNA virus in the alphavirus-like supergroup, encodes two nonstructural replication proteins (140K and 66K), both of which are required for its RNA genome replication. The 140K protein contains domains indicative of methyltransferase, proteinase, and NTPase/helicase activities, while the 66K protein encompasses the RNA-dependent RNA polymerase domain (Jakubiec et al., 2004). The TYMV replication complex is associated with membrane vesicles present at the chloroplast envelope and differs significantly from polymerases of previously studied poliovirus and hepatitis C virus.

The antiviral activity of (*S*)-HPMPC and PMEDAP on ssRNA virus replicating in chloroplasts is surprising. Balzarini et al. (1993) stated that as a rule the HPMP derivatives proved effective against DNA viruses but not RNA viruses or retroviruses. From the ANPs tested in our experiments, only (*S*)-HPMPC and PMEDAP revealed moderate virustatic effects against ssRNA plant virus comparable to, but not better than, that of ribavirin, although this was accompanied by higher phytotoxicity. The facts that (*S*)-HPMPC and PMEDAP are the only ANPs showing some effect on the virus replication and they are also the only ones exhibiting major impact on dry weight of plants and plant survival raises questions as to their selective antiviral effect. We cannot exclude the possibility that the antiviral effect described in this study is due to phytotoxicity.

In contrast to PMEA and (*R*)-PMPA, which apparently have no antiviral effect against ssRNA virus, they remain potential candidates for further testing at lower concentrations and additional plant–virus combinations. In the mammalian system, these nucleotide analogues are phosphorylated by cellular kinases to their diphosphates (analogues of nucleoside 5′-triphosphates) (Krejčová et al., 2000; Horská et al., 2006), which inhibit replicative DNA polymerases (Kramata et al., 1996; Cihlář and Chen, 1997; Birkuš et al., 1999, 2001). This ability is believed to explain the cytostatic activity of the title compounds. For example, PMEDAPpp strongly inhibits pol δ (Kramata et al., 1996), while the diphosphate of cidofovir [(*S*)-HPMPCpp] is a relatively weak competitive inhibitor of DNA polymerases α, δ and ε (Birkuš et al., 2001). These findings lead us to suppose that an interaction of (*S*)-HPMPC and/or PMEDAP with the plant DNA and/or ssRNA replication is also conditioned by their phosphorylation. This process is most probably exclusively intracellular, because we found no phosphorylated derivatives in the media.

Helliot et al. (2003) first tested a concentration scale (10, 25, 50 mg/l) of PMEDAP, PMEA and (*R*)-PMPA against *Banana streak virus* (BSV—genus Badnavirus, containing a circular dsDNA genome) on meristems excised from in vitro banana (*Musa sp.*) during 3 months.

They assumed that the mechanism of the selective antiviral activity of tenofovir, adefovir and PMEDAP on badnavirus replication can thus most likely be ascribed to an inhibition of reverse transcriptase activity although formal proof is still needed to demonstrate an inhibitory effect of the diphosphates of PMPA, PMEA and PMEDAP on the (recombinant) enzyme. An interesting question to address would also be the characteristics of intracellular phosphorylation of the acyclic nucleoside phosphonates in plant cells, because biosynthetic pathways of nucleosides/nucleotides in plant and animal cells are in the principle the same. Several enzymes have been reported to be involved in the phosphorylation of adefovir and tenofovir. These enzymes include 5-phosphoribosyl-1-pyrophosphate (PRPP) synthetase (Balzarini et al., 1991), AMP(dAMP) kinase (Merta et al., 1992; Krejčová et al., 2000) and nucleoside diphosphate kinase (Horská et al., 2006). Plants encode enzymes that are their homologues.

In the literature most authors used virustatics in concentrations of 5–25 mg/l (Verma et al., 2005). Based on our preliminary test with PMEA, we selected 50 mg/ml as the upper limit concentration for observing both antiviral and possible phytotoxic effect of ANPs. Our results on PMEDAP phytotoxicity are similar to those obtained by Helliot et al. (2003). In contrast to our experiments, they found also high toxicity of (*R*)-PMPA (25 mg/l) and somewhat lower toxicity of PMEA (50 mg/l). Higher toxicity of ANPs to more sensitive explants than to the whole plant is not surprising. The different effects of ANPs on *B. pekinensis* in comparison to banana show the necessity to test the phytotoxicity of potent antivirals for particular plant species before their use in practice.

We are aware that the uptake, distribution and metabolism of ANPs in plants are critical for the evaluation of their antiviral activity. We focused, therefore, also on analyzing the change of concentration during the cultivation of plants, which had been neglected by authors testing antivirals on, for example, meristems cultivated on solid media. Although from the practical point of view poor transport of a compound will affect antiviral activity and lead to its exclusion from further tests, this data could help us to understand the mode of action of ANPs in plants. With the exception of (*R*)-PMPA, the concentration of ANPs and ribavirin was decreasing and its variation in some Magenta® vessels was influenced by the evaporation of water from the media. According to Hansen (1989), ribavirin is translocated within 24–48 h, and especially to actively growing parts of the plant. Indirect evidence from other experiments shows that the time course for translocation of compounds of similar molecular size is in the same range. Concentration decrease signals various uptake modes for compounds by the transpiration flow in plants. The comparison of data from TYMV-infected and uninfected plants shows that the translocation was similar. The variation in uptake in some compounds indicated by S.D. in Table 4 could result from different growth of individual plants, evaporation and lower number of analyzed samples. However, a comparison of results in Tables 2 and 4 indicates that the uptake cannot be automatically correlated with the incorporation of antivirals into nucleic acids or antiviral effects in general. Moreover, the compounds were present in surplus amounts in the medium.

In conclusion, we first report an antiviral potency of the ANPs (*S*)-HPMPC and PMEDAP on an RNA virus. Second, we have proven that a method of cultivating whole plants on liquid medium can join those methods already used (Hansen, 1989) that are effective in selecting potential antivirals against plant viruses. In our opinion, it reflects better the real inhibitory effect of tested compounds upon systemic infection and is more convenient for practical use in eliminating viruses from infected explants under in vitro conditions.

Acknowledgements

This project was funded by grants nos. 522/09/0707 from the Grant Agency of the Czech Republic, AV0Z50510513 from the Czech Academy of Sciences and by research project OZ40550506 of the Institute of Organic Chemistry and Biochemistry. The authors thank Dr. I. Dostálková, Ph.D., Faculty of Science, University of South Bohemia, for consultations on statistical processing of the data.

References

Awan, A.R., Mughal, S.M., Iftikhar, Y., Khan, H.Z., 2007. *In vitro* elimination of potato leafroll polerovirus from potato varieties. Eur. J. Sci. Res. 18, 155–164.

Balzarini, J., Hao, Z., Herdewijn, P., Johns, D.G., De Clercq, E., 1991. Intracellular metabolism and mechanism of anti-retrovirus action of 9-(2-phosphonylmethoxy-ethyl)adenine, a potent anti-human immunodeficiency virus compound. Proc. Natl. Acad. Sci. U.S.A. 88, 1499–1503.

Balzarini, J., Holý, A., Jindrich, J., Naesens, L., Snoeck, R., Schols, D., De Clercq, E., 1993. Differential antiherpesvirus and antiretrovirus efects of the (*S*) and (*R*) enantiomers of acyclic nucleoside phosphonates: potent and selective in vitro and in vivo antiretrovirus activities of (*R*)-9-(2-phosphonomethoxypropyl)-2,6-diaminopurine. Antimicrob. Agents Chemother. 37, 332–338.

Birkuš, G., Votruba, I., Holý, A., Otová, B., 1999. 9-[2-(Phosphonomethoxy)ethyl]adenine diphosphate (PMEApp) as a substrate toward replicative DNA polymerases alpha, delta, epsilon, and epsilon. Biochem. Pharmacol. 58, 487–492.

Birkuš, G., Votruba, I., Otmar, M., Holý, A., 2001. Interactions of 1-[(*S*)-3-hydroxy-2-(phosphonomethoxy)propyl]cytosine (cidofovir) diphosphate with DNA polymerases α, δ and ε*. Collect. Czech. Chem. Commun. 66, 1698–1706.

Birkuš, G., Hájek, M., Kramata, P., Votruba, I., Holý, A., Otová, B., 2002. Tenofovir diphosphate is a poor substrate and a weak inhibitor of rat DNA polymerases α, δ and ε*. Antimicrob. Agents Chemother. 46, 1610–1613.

Büchen-Osmond, C. (Ed.), 2006. ICTVdB—The Universal Virus Database, version 4. ICTVdB Management, Mailman School of Public Health, Columbia University, New York, NY.

Cihlář, T., Chen, M.S., 1997. Incorporation of selected nucleoside phosphonates and anti-human immunodeficiency virus nucleotide analogues into DNA by human DNA polymerases alpha, beta and gamma. Antiviral Chem. Chemother. 8, 187–195.
Clark, M.F., Adams, A.N., 1977. Characteristics of the microplate method for enzyme-linked immunosorbent assay for the detection of plant viruses. J. Gen. Virol. 34, 475–483.
De Clercq, E., 2007. The acyclic nucleoside phosphonates from inception to clinical use: historical perspective. Antiviral Res. 75, 1–13.
Faccioli, G., Colombarini, A., 1996. Correlation of potato virus S and virus M contents of potato meristem tips with the percentage of virus-free plantlets produced in vitro. Potato Res. 39, 129–140.
Francki, R.I., Matthews, R.E., 1962. Some effects of 2-thiouracil on the multiplication of turnip yellow mosaic virus. Virology 17, 367–380.
Hansen, A.J., Lane, W.D., 1985. Elimination of apple chlorotic leafspot virus from apple shoot cultures by ribavirin. Plant Dis. 69, 134–135.
Hansen, A.J., 1989. Antiviral chemicals for plant disease control. Crit. Rev. Plant Sci. 8, 45–88.
Helliot, B., Panis, B., Frison, E., De Clercq, E., Swennen, R., Lepoivre, P., Neyts, J., 2003. The acyclic nucleoside phosphonate analogues, adefovir, tenofovir and PMEDAP, efficiently eliminate banana streak virus from banana (*Musa* ssp.). Antiviral Res. 59, 121–126.
Holý, A., Rosenberg, I., Dvořáková, H., 1989. Synthesis of N-(2-phosphonylmethoxyethyl) derivatives of heterocyclic bases. Collect. Czech. Chem. Commun. 54, 2190–2210.
Horská, K., Votruba, I., Holý, A., 2006. Interaction of phosphates of the acyclic nucleoside phosphonates with nucleoside diphosphate kinase from yeast and bovine liver. Collect. Czech. Chem. Commun. 71, 35–43.
James, D., Trytten, P.A., Mackenzie, D.J., Towers, G.H.N., French, C.J., 1997. Elimination of apple stem grooving virus by chemotherapy and development of immunocapture RT-PCR for rapid sensitive screening. Ann. Appl. Biol. 131, 459–470.
Jakubiec, A., Notaise, J., Tournier, V., He'ricourt, F., Block, M.A., Drugeon, G., van Aelst, L., Jupin, I., 2004. Assembly of *turnip yellow mosaic virus* replication complexes: interaction between the proteinase and polymerase domains of the replication proteins. J. Virol. 78, 7945–7957.
Kramata, P., Votruba, I., Otová, B., Holý, A., 1996. Different inhibitory potencies of acyclic phosphonomethoxyalkyl nucleotide analogs toward DNA polymerases alpha, delta and epsilon. Mol. Pharmacol. 6, 1005–1011.
Krejčová, R., Horská, K., Votruba, I., Holý, A., 2000. Phosphorylation of purine (phosphonomethoxy)alkyl derivatives by mitochondrial AMP kinase (AK2 type) from L1210 cells. Collect. Czech. Chem. Commun. 65, 1653–1668.
Lerch, B., 1987. On the inhibition of plant virus multiplication by ribavirin. Antiviral Res. 7, 257–270.
Lim, S.T., Wong, S.M., Goh, C.J., 1993. Elimination of cymbidium mosaic virus and odontoglossum ringspot virus from orchids by meristem culture and thin section culture with chemotherapy. Ann. Appl. Biol. 122, 289–297.
Merta, A., Vortruba, I., Jindrich, J., Holý, A., Cihlár, T., Rosenberg, I., Otmar, M., Herve, T.Y., 1992. Phosphorylation of 9-(2-phosphonomethoxyethyl)adenine and 9-(*S*)-(3-hydroxy-2-phosphonomethoxypropyl)adenine by AMP (dAMP) kinase from L1210 cells. Biochem. Pharmacol. 17, 2067–2077.
Murashige, T.F., Skoog, F., 1962. A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiol. Plant. 15, 473–497.
Naesens, L., Snoeck, R., Andrei, G., Balzarini, J., Neyts, J., De Clercq, E., 1997. HPMPC (cidofovir), PMEA (adefovir) and related acyclic nucleoside phosphonate analogues: a review of their pharmacology and clinical potential in the treatment of viral infections. Antiviral Chem. Chemother. 8, 1–23.
Panattoni, A., D'Anna, F., Cristani, C., Triolo, E., 2007a. Grapevine vitivirus A eradication in Vitis vinifera explants by antiviral drugs and thermotherapy. J. Virol. Methods 146, 129–135.
Panattoni, A., D'Anna, F., Triolo, E., 2007b. Antiviral activity of tiazofurin and mycophenolic acid against Grapevine leafroll-associated virus 3 in Vitis vinifera explants. Antiviral Res. 73, 206–211.
Parker, W.B., 2005. Metabolism and antiviral activity of ribavirin. Virus Res. 107, 165–171.
Petrzik, K., 2009. Occurrence of potato virus X on hybrid dock in Czech Republic. Acta Virol. 53, 49–52.
Polák, J., Špak, J., 1987. The incidence of two serologically distinct strains of turnip yellow mosaic virus on cultivated cruciferous plants in Czechoslovakia. Arch. Phytopathol. Pfl. 23, 269–274.
Quecini, V., Lopes, M.L., Pacheco, F.T.H., Ongarelli, M.D.G., 2006. Ribavirin, a guanosin analogue mammalian antiviral agent, impairs tomato spotted wilt virus multiplication in tobacco cell cultures. Arch. Phytopathol. Plant Prot. 41, 1–13.
Spiegel, S., Frison, E.A., Converse, R.H., 1993. Recent developments in therapy and virus-detection procedures for international movement of clonal plant germ plasm. Plant Dis. 77, 1176–1180.
Stahl, C., Sander, E., 1992. Inhibition of potato virus X (PVX) synthesis in tobacco protoplasts by ethidium bromide as standard: a possible screening system for antiviral substances and for transfer of virus resistance genes. J. Plant Dis. Prot. 99, 449–459.
Suo, Z., Johnson, K.A., 1998. Selective inhibition of HIV-1 reverse transcriptase by an antiviral inhibitor, (R)-9-(2-phosphonylmethoxypropyl)adenine. J. Biol. Chem. 273, 27250–27258.
Sutula, C.L., Gillett, J.M., Morrissey, S.M., Ramsdell, D.C., 1986. Interpreting ELISA data and establishing the positive–negative. Plant Dis. 70, 722–726.
Verma, N., Ram, R., Zaidi, A.A., 2005. In vitro production of Prunus necrotic ringspot virus-free begonias through chemo- and thermotherapy. Sci. Hortic. 103, 239–247.
Xia, Y., Fan, Z., Yao, J., Liao, Q., Li, W., Qu, F., Peng, L., 2006. Discovery of bitriazolyl compounds as novel antiviral candidates for combating the tobacco mosaic virus. Bioorg. Med. Chem. Lett. 16, 2693–2698.