Pergamon

PII: S0031-9422(95)00522-5

Phytochemistry, Vol. 43, No. 6, pp. 1271–1276, 1996

0031-9422/96 $15.00 + 0.00

INHIBITION OF TOMATO RINGSPOT VIRUS BY FLAVONOIDS

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(*Received in revised form* 21 *June* 1996)

Key Word Index—Flavonoids; tomato ringspot; nepovirus; anti-viral; *Chenopodium*.

Abstract—When applied in a mixed inoculum with tomato ringspot nepovirus (TomRSV), flavonoids and related compounds inhibited infectivity in *Chenopodium quinoa*. Compounds that showed strong anti-viral activity were: quercetin, quercetin 3-methyl ether, quercetin 7-methyl ether, quercetin 3,7,3′4′-tetramethyl ether, galangin 3-methyl ether, morin, robinin, quercetin 3,7,4′-trimethyl ether, quercetin 7,4′-dimethyl ether, 7,4′-di-*O*-benzol-quercetin, 7-hydroxy-3,4′-dimethyl flavone, 6,3′-dihydroxy-4′-methyl aurone and fisetin 4′-methyl ether. Quercetin applied at a concentration of 5 μg ml^{-1} caused 70% inhibition of local lesion development. When quercetin was applied to leaves prior to inoculation, there was only slight induced resistance to infection. Quercetin at 5 μg ml^{-1} did not affect virus multiplication in protoplasts prepared from cucumber cotyledons and transfected with viral-RNA. In meristematic tip cultures, quercetin reduced virus titre by up to 89% over a period of 36 weeks whereas ribavirin caused a 25% reduction over the same period. It is proposed that flavonoids interfere with an early event in the virus life cycle resulting in decreased infectivity and titre in tissue culture. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

Flavonoids are low-molecular-mass compounds widely distributed in the plant kingdom [1]. Although their presence in plants has been known for many years, they were originally considered unimportant. Many biological activities are now associated with these compounds, including anti-viral activity against a range of animal viruses such as poliovirus [2], herpes simplex virus [3] and pseudorabies virus [4]. In addition, they play an important role in the interaction between plants and their environment [1].

Antiviral activity of flavonoids against a plant virus, potato virus X (PVX) was first reported by Verma [5]. Several years later, the effects of a range of flavonoids on tobacco mosaic virus (TMV) [6] and potato virus X (PVX) [7] were studied. Flavonoids reduced TMV infectivity, apparently by weakening interactions between coat protein sub-units, leading to increased susceptibility to host RNAses [6]. It was proposed that quercetin interferes with an interaction between PVX coat protein and a host component required for infection [7]. It was evident from previous studies [6, 7] that flavonoid structure/activity relationship is very important in determining the anti-viral activity.

We have continued to screen a wider range of compounds using tomato ringspot virus (TomRSV) and thus extended prevous studies to the nepovirus group. We also explored whether induced resistance within the host plant can be activated by application of such compounds. The effects of flavonoids on virus multiplication in plant protoplasts and infected tissue cultures were also assessed.

RESULTS AND DISCUSSION

Several flavonoids and related compounds were found to have anti-viral activity against TomRSV (Table 1). Quercetin, quercetin 7,4′-dimethyl ether, quercetin 3,7,4′-trimethyl ether and fisetin 4′-methyl ether were the most effective compounds, causing 67 to 76% inhibition of TomRSV infectivity when applied at 10 μg ml^{-1} (Table 1).

The following compounds were tested at 10 and 50 μg ml^{-1} and found to have little or no activity against TomRSV infectivity (less than 10% inhibition): quercetagetin (3,5,6,7,3′,4′-hexahydroxyflavone), kaempferol (3,5,7,4′-tetrahydroxyflavone), flavone 3-methyl ether, acacetin (5,7-dihydroxy-4′-methoxy-

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Table 1. Inhibition of tomato ringspot infectivity by flavonoids and related compounds in *Chenopodium quinoa*

Compound	Concentraton ($\mu g\ ml^{-1}$) 10	50
	Inhibition (%)	
Morin (3,5,7,2′,3′-pentahydroxyflavone)	28±1.4	34±0.8
Quercetin (3,5,7,3′,4′-pentahydroxyflavone)	73±2.2	56±1.9
Quercetin 7-methyl ether	ND	76±5.5
Quercetin 3-methyl ether	56±2.8	55±4.1
Quercetin 7,4′-dimethyl ether	76±6.2	73±7.5
Quercetin 3,7,4′-trimethyl ether	67±7.3	67±2.3
Quercetin 3,7,3′,4′-tetramethyl ether	51±3.6	66±1.5
Dihydroquercetin	23±2.2	29±2.6
Galangin 3-methyl ether	47±3.0	50±1.8
Robinin (kaempferol 3-robinobioside-7-rhamnoside)	39±4.1	54±6.2
7-Hydroxy-3,4′-dimethylflavone	64±6.3	68±5.4
3-Hydroxyflavone	16±2.3	ND
Dihydroflavone	38±4.5	34±2.0
6-Hydroxy 4,3′-dimethylaurone	22±0.8	ND
2-Hydroxychalcone	30±2.6	ND
6,3′-Dihydroxy 4′-methyl aurone	46±5.2	ND
4′-Methyl fisetin (3,7,3′,4′-tetrahydroxyflavone)	68±1.9	ND
Galangin (3,5,7-trihydroxyflavone)	ND	45±2.2
Luteolin (5,7,3′,4′-tetrahydroxyflavone)	ND	38±2.3
Sulfuretin (6,3′,4′-trihydroxyaurone)	ND	59±4.9
Fustin (2,3-dihydrofisetin)	31±4.3	ND
7,4′-Di-*O*-benzyolquercetin	ND	56±3.8

Results are means ±SE of three separate experiments and all values are significant at $p = 0.05$. ND: not determined.

flavone), catechin (3,5,7,3′,4′-pentahydroxyflavan), primuletin (5-hydroxyflavone), pelargonidin chloride (3,5,7,4′-tetrahydroxyflavylium chloride), apigenin-7,4′-disulphate, kaempferol-7,4′-disulphate, 2′-hydroxylupalligenin, apigenin (5,7,4′-trihydroxyflavone), genistein (5,7,4′-trihydroxyisoflavone) and 2′-hydroxygenistein.

In earlier studies [7], quercetin and derivatives effectively inhibited infectivity of PVX; however, quercetin had no effect against TMV [6]. In the present study, quercetin also showed strong anti-viral activity against TomRSV. From our results (Table 1), it may be observed that a pentahydroxyflavone, as exemplified in the quercetin structure, forms an ideal model for anti-TomRSV activity. Deviation from this basic structure either had no significant effect or generally decreased the inhibitory activity (Table 1). Thus, the addition of an extra hydroxy group at C-6 (quercetagetin) totally eliminated inhibition. This agrees with the findings of French and Towers [7] on the effect of quercetagetin on PVX infectivity. Again, hydroxylation of C-2′ (morin) compared with 4′ (quercetin) reduced inhibition at both 10 and 50 $\mu g\ ml^{-1}$. *O*-Methylation of quercetin at various positions had varying effects on anti-viral activity. Substitution of a methoxy group at C-3 reduced inhibition to 56% when compared to quercetin at a concentration of 10 $\mu g\ ml^{-1}$. This is in contrast to the finding of French *et al.* [6], that *O*-methylation at position 3 was required for inhibition of TMV. On the other hand, when additional *C*-atoms were methoxylated as in quercetin 3,7,4′-trimethyl ether and 3,7,3′,4′-tetramethyl ether, there was little effect on activity, compared to quercetin. Similarly, when the activity of quercetin 7,4′-dimethyl ether (76% inhibition) was compared with the activity of pure quercetin aglycone (73%) at 10 $\mu g\ ml^{-1}$, it was again observed that *O*-methylation did not significantly affect the inhibition of TomRSV. This further demonstrates that methoxylation of quercetin was not required for inhibition of TomRSV.

Kaempferol was ineffective against TomRSV but when glycosylated as in robinin, caused 54% inhibition. This difference may be due to relative solubility since the glycoside is much more water-soluble than the aglycone. Also since kaempferol caused significant inhibition of PVX [7], and as both PVX and TomRSV were used to infect *Chenopodium quinoa*, this observed difference in inhibition cannot be attributed to host factors. Inhibition of viruses by flavonoids is clearly virus-specific and very dependent on substitution patterns in the molecule. Of the three viruses studied to date, PVX and TomRSV showed the most similar inhibition patterns when treated with the same test compounds (exceptions are noted above).

Inhibition of TomRSV infectivity by quercetin could be due to an effect within the cell or an effect of virus particles from outside the plasma membrane. To assess the role of the viral coat protein, quercetin and purified viral RNA were used in a mixed inoculum. There was no inhibitory effect of quercetin when used in combina-

tion with RNA but rather there was an increase (54%) in TomRSV infectivity. This shows that the inhibitory effect of quercetin is dependent on the presence of the coat protein for anti-viral activity. For example, quercetin may be exerting the inhibitory effect by an interaction with the virus particle, possibly by inhibition of uncoating. Such a mechanism was suggested for the interaction between the synthetic flavonoid Ro 09-0179 (5,4′-dihydroxy-3,7,3′-trimethoxyflavone) and rhinoviruses [8].

Time course experiments revealed only a slight (23%) induced inhibition of TomRSV infectivity when quercetin was applied 48 hours before inoculation (Fig. 1). Maximum inhibition (70%) was conferred when quercetin was applied together with TomRSV in a mixed inoculum. Application of quercetin on leaves just before and after inoculation with TomRSV did not cause any inhibition of infectivity. These results were similar to the pattern of inhibition of quercetin with PVX [7].

To assess the effectiveness of quercetin in inhibiting virus replication, the effects of quercetin were assessed in isolated protoplasts prepared from sterile cucumber cotyledons. When viral RNA was used to transfect protoplasts, there was no inhibition of virus multiplication by quercetin. This shows that quercetin does not inhibit viral replication. This finding is consistent with the lack of inhibition of virus by quercetin when applied to whole plants after TomRSV inoculation (Fig. 1).

In tissue culture experiments, the titre of TomRSV was decreased in cultures treated with quercetin compared to the controls (Fig. 2). Virus titre decreased in treated cultures at three concentrations of quercetin (0.1, 1.0 and 10 $\mu g\,ml^{-1}$ in culture medium) as the duration of treatment increased. After 36 weeks of maintaining cultures in the presence of quercetin, TomRSV titres were reduced by up to 89% (Fig. 2). Virus titre fluctuated throughout the period in controls as well as in treated cultures. At the end of the experiment, there was a greater reduction of TomRSV titre by quercetin at 0.1 $\mu g\,ml^{-1}$ and 10 $\mu g\,ml^{-1}$ compared with 1 $\mu g\,ml^{-1}$. The reason for this is not known but may be related to differences in periodicity of the virus titre in the three separate tissue cultures.

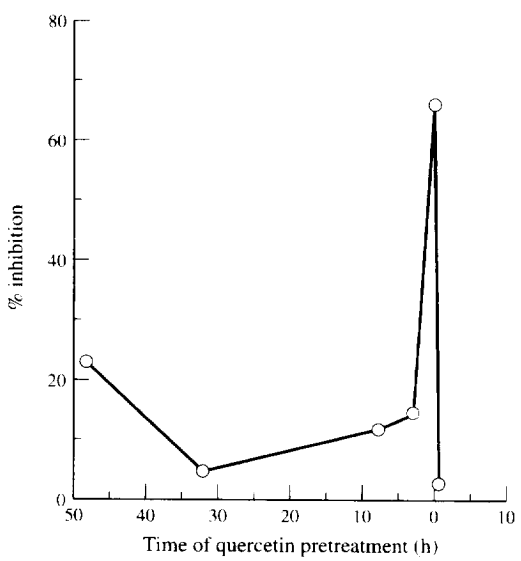

Fig. 1. Quercetin was applied at 5 $\mu g\,ml^{-1}$ to leaves of *Chenopodium quinoa* at 48, 32, 8, 3 and 0 hr before and 0.5 hr after inoculation with TomRSV and infectivity was calculated by local lesion assay. The experiment was repeated three times.

Virus replication from viral RNA was not inhibited by quercetin, as judged by transfected protoplasts, and yet virus titre gradually decreased in tissue culture. One possible explanation is an inhibition of virus movement by quercetin. TomRSV is thought to move by the formation of tubular structures through which intact virus particles are transported from cell-to-cell [9, 10]. Therefore, in each successive infected cell the virus must undergo a complete life-cycle including uncoating of the particle, before virus replication can proceed. Since quercetin interferes with an early event in the virus life cycle [7], for example binding to the viral coat protein and/or interfering with uncoating of the virus [8], this could account both for the inhibition of infectivity of the intact virus in whole plants and the gradual reduction of virus titre in tissue cultures. Also, if inhibition were acting prior to the production of viral RNA, it would be expected that the compounds would not affect infectivity using viral RNA in whole plants. This was observed here and previously [7].

Ribavirin is a broad-spectrum, anti-viral compound that has been used against a variety of animal and plant viruses [11], although the utility of this compound is limited by cellular toxicity [12]. Therefore, it was of interest to compare quercetin with ribavirin in their ability to reduce virus concentration in infected tissue cultures. Ribavirin was also effective against TomRSV but to a lesser extent (Fig. 3); 10 $\mu g\,ml^{-1}$ caused 25% inhibition after 33 weeks in tissue culture. It is evident that quercetin was a more effective inhibitor than ribavirin in *N. occidentalis* tissue culture. Also, quercetin has the advantage of low cytotoxicity and cost. Thus, flavonoids are shown to be another class of compound with potent activity against plant viruses and have the potential to be used to reduce or eliminate viruses from clonal plant material.

EXPERIMENTAL

Virus source and purification. Tomato ringspot virus was obtained from the plant virus collection at the Pacific Agriculture Research Centre, Vancouver. The virus was maintained and purified from *Nicotiana clevelandii* using a slightly modified method of Stace-Smith [13]. Purified virus was checked for the presence of any contaminants using the background staining technique and viewing under the transmission electron microscope (Hitachi 7000). Any trace of flavonoids was also checked. Three separate thin spots of the purified virus preparation were dried on a cellulose

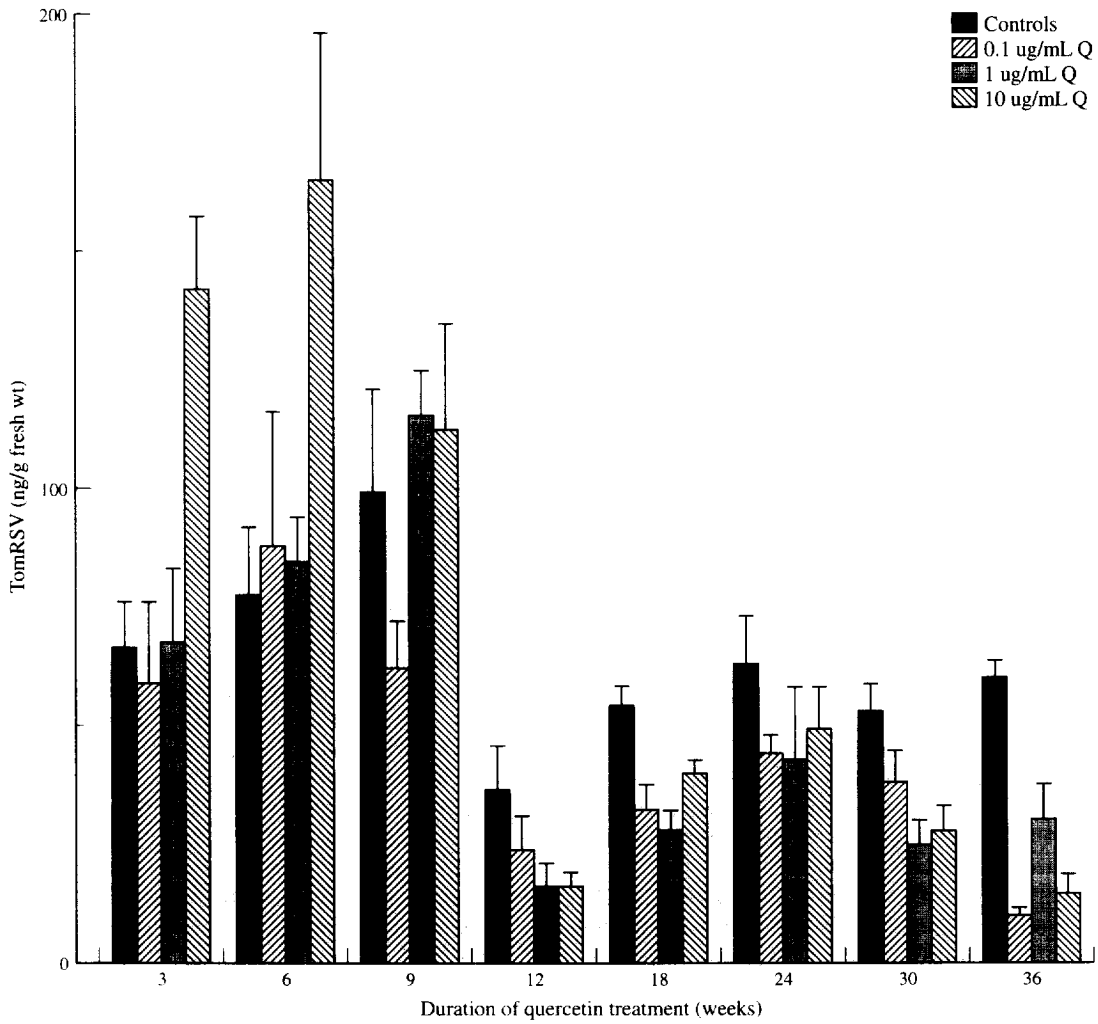

Fig. 2. Effect of quercetin on *Nicotiana occidentalis* tissue culture. The culture was initiated and maintained using MS liquid medium. Quercetin concentration was maintained in the range from 0.1 to 10 $\mu g\ ml^{-1}$ for 36 weeks. Plant tissue was sampled at different durations and concentrations of TomRSV were determined by ELISA. Each value is an average of eight replicates.

plate (without fluorescent indicator) and viewed under UV/NH_3 for presence of any dark/yellow colours. In all cases, absence of flavonoids was observed. Concentration of pure TomRSV was determined spectrophotometrically using standard methods [13].

Plant material and local lesion assay. Chenopodium quinoa L. seedlings were grown for 6 weeks in a greenhouse on a 16/8 hr day/night cycle at 20–25°. Leaves were dusted with carborundum and purified TomRSV was mechanically inoculated onto half leaves of *Chenopodium quinoa* as described previously [7]. Each treatment was replicated on 12 half leaves and experiments were repeated 3×. Each compound was dissolved in ethylene glycol monomethylether (EGME) at a concn of 2.5 mg ml^{-1} and stored at −20°. Aliquots were diluted with NaH_2PO_4 buffer (10 mM, pH 7.2) to give final concentrations. Prior to inoculation, TomRSV was incubated for 5 min at 25° with the compounds. Controls contained identical concentrations of buffer and EGME. At this concentration, EGME did not affect the number of lesions produced by TomRSV. In some experiments purified viral RNA (100 $\mu g\ ml^{-1}$) was mixed with quercetin (5, 10 and 50 $\mu g\ ml^{-1}$) and applied in a mixed inoculum. Infectivity was calculated by half-leaf local lesion assays, as above. Local lesions were counted 5 days after virus infection.

Time course experiment with whole plants. Quercetin at 5 $\mu g\ ml^{-1}$ was applied with gloved fingers to leaves of whole plants of *Chenopodium quinoa* 48, 24, 8, 3 and 0 hr before and 0.5 hr after TomRSV inoculation. In parallel experiments, quercetin was also applied to half leaves before and immediately after inoculation. Control plants or half leaves were treated with EGME in NaH_2PO_4 buffer. Leaves were rinsed with tap water and air dried to wash off the excess compound on the leaf surface before inoculating with TomRSV. Local lesions were counted after 5 days.

Protoplast isolation and transfection with TomRSV viral-RNA. Protoplasts were isolated from *Cucumis sativus* L. cotyledons according to the method of

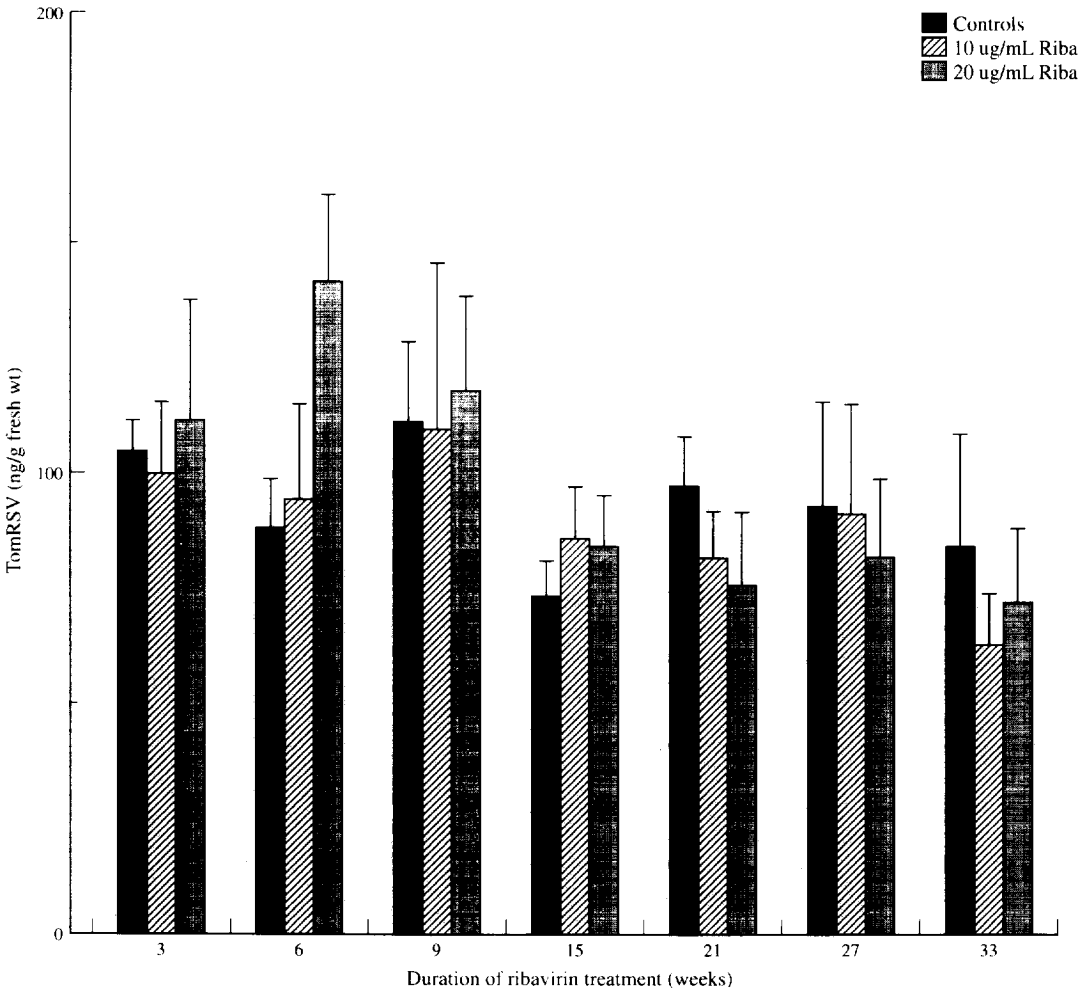

Fig. 3. Effect of ribavirin on *Nicotiana occidentalis* tissue culture. The culture was initiated and maintained using MS liquid medium. Ribavirin concentration was maintained in the range from 10 to 20 μg ml^{-1} for 33 weeks. Plant tissue was sampled at different durations and concentrations of TomRSV were determined by ELISA. Each value is an average of eight replicates.

Wieczorek and Sanfaçon [14]. Protoplasts were counted with a hemocytometer (Neubauer) and transfected with virus particles or isolated RNA in the presence of polyethylene glycol [14, 15].

TomRSV-RNA was isolated from purified TomRSV particles by using phenol/chloroform in the presence of 1 M Tris–HCl, pH 8.9, 20% SDS and 10 mM EDTA. The RNA pellet was washed with 70% ethanol and resuspended in cold, sterile deionized water at a concentration of 1 μg ml^{-1} and stored at −70°. Protoplasts were transfected according to a previous method [14]. Immediately before transfection, protoplasts were transferred to MaCa medium and the concentration adjusted to 2×10^6 viable protoplasts ml^{-1}. To transfect the protoplasts, 10 μg of purified virus or viral RNA was mixed with 0.3 ml protoplasts and an equal volume of 40% PEG 3250 (Sigma) solution was added. After adding PEG, protoplasts were immediately diluted with 10 ml of Cucumber Medium 1 (CMI) and transferred to ice for 15 min. Quercetin at 5 μg ml^{-1} was added immediately before viral RNA. Controls were conducted by transfecting protoplasts in the absence or presence of RNA, without quercetin (protoplast − RNA or protoplast + RNA). An additional control, consisting of protoplast + quercetin, was conducted to assess phytotoxicity of quercetin. Protoplasts were then centrifuged at 700 r.p.m. for 5 min and the pellet was washed with CMI and resuspended in 5 ml of CMI in polypropylene tubes. Tubes were incubated at 20–25° under a 16/8 hr day/night cycle (200 μmol m^{-2} s^{-1}). After 40 hr of incubation, protoplasts were centrifuged, washed with incubation buffer and pelleted again. The pellet was resuspended in distilled water to rupture protoplasts. Alternatively, pelleted protoplasts were resuspended in NaH_2PO_4 buffer (10 mM, pH 7.2) and sonicated for 30 sec. Both methods were equally efficient in disrupting protoplasts as determined by light microscopy. Disrupted protoplasts treated with quercetin-RNA and viral RNA alone (controls) were used to inoculate *Chenopodium quinoa*

and per cent inhibition was calculated from the local lesion assay.

Effect of quercetin and ribavirin in tissue culture. Plants of *Nicotiana occidentalis*, healthy and infected with TomRSV were initiated from apical meristems in separate cultures and proliferated using a medium consisting of Murashige and Skoog salts [16] and supplemented with (per litre) 100 mg inositol, 80 mg adenine sulphate, 0.4 mg thiamine-HCl, 30 g sucrose, 18 mg ascorbic acid and 2 mg zeatin. The medium was adjusted to pH 5.7, and Difco Bacto agar was added to 0.8%, prior to autoclaving.

A range of concentrations (0, 0.25, 0.5, and 1 $\mu g\,ml^{-1}$) of EGME and dimethyl sulphoxide (DMSO) were assessed for phytotoxic effects when added to medium for the proliferation of *Nicotiana occidentalis*. In contrast to whole plants and protoplasts, EGME (0.25–1.0 $\mu g\,ml^{-1}$) caused symptoms of phytotoxicity in plants grown on liquid or solid medium. Plants growing on media containing DMSO appeared vigorous and healthy. DMSO (0.1% final concentration) was therefore selected as the solvent for dissolving the flavonoids in tissue culture application.

Quercetin was maintained at concentrations in the range from 0.1 to 10 $\mu g\,ml^{-1}$ of liquid medium. Samples were collected for 36 weeks and concentrations of TomRSV were determined by quantitative ELISA. The effects of another antiviral compound, ribavirin, were also studied in tissue culture at concentrations of 10 and 20 $\mu g\,ml^{-1}$ for a period of 33 weeks. Concentrations of TomRSV were expressed as $ng\,g^{-1}$ fresh weight of tissue.

Quantitative determination of TomRSV. Known quantities of purified TomRSV were diluted in healthy *N. occidentalis* sap. A triple antibody sandwich ELISA was used for the detection of TomRSV [17]. This used a TomRSV polyclonal antibody for trapping, a TomRSV monoclonal as the secondary antibody, and a goat anti-mouse alkaline phosphatase IgG + IgM conjugate. Eight replicates of each virus concentration were placed in wells of a microtitre plate. The average absorbance values of readings taken at 405 nm after 15 min, were calculated for each virus concentration and used to plot a graph which was used to determine TomRSV concentration after treatment with the various flavonoids.

Acknowledgements—We thank the Natural Sciences and Engineering Research Council of Canada for financial assistance in the form of a Strategic Grant. We also thank Dr C. A. Williams of Reading University, U.K. for providing some of the flavonoids used in this research.

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