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Short communication

Inhibition of tomato bushy stunt virus infection using a quercetagetin flavonoid isolated from *Centaurea rupestris* L.

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Abstract

Flower and leaf extracts of *Centaurea rupestris* L. and also the flavonoid quercetagetin 3′-methylether isolated from these extracts revealed a strong antiviral activity when inoculated simultaneously with tomato bushy stunt virus in two *Nicotiana* species. Almost complete reduction of local lesion number resulted from these inoculations in *N. glutinosa*. A similar effect was observed in inoculated leaves of *N. megalosiphon* causing the absence of systemic infection in 40% of treated plants. The antiphytoviral activity was not a consequence of induced resistance, inhibition of virus multiplication, suppression of symptom development or direct virus inactivation. Results presented in this paper suggest that the flavonoid may interfere with the initiation of virus infection. © 1997 Elsevier Science B.V.

Keywords: Centaurea rupestris L.; Flavonoid; Antiviral activity; Plant virus

1. Introduction

Although there has been much progress in basic and applied research in understanding the control of replication of human and animal viruses, few compounds, identified as animal antiviral substances, have been examined for their antiphytoviral activity (Hansen, 1988; Schuster, 1988). Several of these antiviral chemicals may potentially be used in the control of viral diseases of important crops in agriculture. Virazole (ribavirin) and some related synthetic nucleoside analogues were found to possess antiphytoviral activity (De Fazio et al., 1980; Caner et al., 1984; Dawson and Lozoya-Saldana, 1984; Nagy et al., 1989). Other compounds have also been identified which inhibit virus replication at doses that are not toxic to the host plant (Schuster and Holý, 1988; Schuster et al., 1989; De Fazio et al., 1990).

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A number of flavonoid compounds inhibit either infection of cells or replication of some animal viruses. Some of these naturally occurring and widely distributed plant substances inhibit the reverse transcriptase activity of several retroviruses (Spedding et al., 1989; Ono et al., 1990; Baylor et al., 1992). Some reports describe the use of flavonoids as potent and selective inhibitors of viral RNA synthesis and antisialidase activity in poliovirus- and influenzavirus-infected cells, respectively (Gonzales et al., 1990; Nagai et al., 1990, 1992). Flavonoids have been found to possess a broad-spectrum antimicrobial activity against many microorganisms, including bacteria, fungi and yeasts (Barnabas and Nagarajan, 1988; Tomas-Barberan et al., 1988).

A few studies have demonstrated the antiphytoviral activity of flavonoids. A range of flavonoids inhibit the infection by tobacco mosaic virus and potato virus X (Verma, 1973; French et al., 1991; French and Towers, 1992). The efficiency of virus inhibition depended on the way the flavonoids were applied and the proposed mode of inhibition varied according to the flavonoid/virus/host combination studied. In this study, a flavonoid (quercetagetin 3′-methylether) was isolated from *Centaurea rupestris* L. and analyzed for its antiviral activity against tomato bushy stunt virus in two *Nicotiana* species.

2. Material and methods

2.1. Extraction and analysis of the flavonoid

Crude flavonoid extracts of leaves and flowers of *Centaurea rupestris* L. (*Asteraceae*) were prepared by percolation in 45% and 90% ethanol and ether (1:1, w/v). The pure flavonoid was isolated from a methanolic extract of flowers by column and preparative thin-layer chromatography (Poole and Poole, 1991). Thin-layer chromatography (TLC) was used to determine the purity of the isolated compound, and also for comparing it with an authentic sample of quercetin (Sigma). These analyses were performed using commercial plates (Merck, TLC Aluminium sheets, silica gel F254) and the solvent systems ethyl acetate–formic acid–acetic acid–water (100:11:11:27) and ethyl acetate–formic acid–water (8:1:1). The spots on the chromatograms were detected by successive spraying with NST reagent and ethanolic solution of polyethylene glycol 4000 (Wagner et al., 1983) and were visualized under UV-light (360 nm).

Investigations of the chemical structure of the isolated flavonoid performed by Dr Neil Robinson (Natural Products Chemistry, Xenova, Slough, UK) showed that it is 3,4′,5,6,7-pentahydroxy-3′-methoxyflavone, i.e. quercetagetin 3′-methylether.

2.2. Virus and plant hosts

Tomato bushy stunt virus (TBSV), type strain (Smith, 1935), was propagated in *Nicotiana megalosiphon* which was chosen as a systemic host. Systemically infected leaves were ground in 0.06 M phosphate buffer, pH 7.0 (1:1, w/v) and centrifuged at low speed to prepare the virus inoculum. This crude sap preparation was diluted with inoculation buffer to yield 80–90 lesions per leaf on *N. glutinosa* which is a local lesion host for this virus. Leaves were dusted with carborundum before virus inoculation.

2.3. Flavonoid application to host plants

Crude extracts of *C. rupestris* leaves and flowers or purified flavonoid preparations were added to the virus inocula or were inoculated 1 h before or 30 s after the virus on *N. glutinosa* leaf halves. The other halves were rubbed with the control inocula in which the virus concentration was adjusted, using inoculation buffer, to that of the test inocula. Three days later the local lesions were counted. The flavonoid was also applied, 2 days before inoculation, by immersing the root system of *N. megalosiphon* or the leaf petioles (*N. glutinosa*) in the flavonoid preparations.

The symptom expression and development of the systemic infection on *N. megalosiphon* were also monitored. For this purpose four groups of host plants (10 plants in each group) were inoculated (two leaves on each plant) with: (a) inoculation buffer; (b) virus; (c) flavonoid; or (d) virus + flavonoid.

For application to the host plants, crude extracts and purified flavonoid preparations were dissolved in virus inoculation buffer. In the virus inocula, as well as in the solution for leaf disc incubation (see below), the flavonoid concentration was adjusted to 1 mg/ml. To apply the flavonoid to the roots or leaf petioles, a crude 45%-ethanol extract from flowers was used, diluted 1:5 with water.

2.4. Leaf disc incubation test

To evaluate the effect of the flavonoid extracted on the virus multiplication, expanded leaves of *N. megalosiphon* were inoculated with the virus and assessed for virus replication in a leaf disc incubation test (De Fazio et al., 1990). The virus content of the leaf discs was determined by a bioassay on *N. glutinosa*. The inhibition percentage (IP) was calculated using the formula $IP = 100 - A/B \times 100$, in which A represents the average local lesion number obtained from the treated discs, and B the corresponding value obtained from the control discs floated on distilled water.

2.5. Virus purification and density gradient centrifugation

To determine the influence of the flavonoid on the virion itself, TBSV was purified from infected leaves of *N. megalosiphon* using the method of Hollings (1962). A sample (0.9 ml) of partially purified virus preparation was mixed with the purified flavonoid (0.1 ml) and centrifuged in a sucrose density gradient (10–40%) at 60 000 × *g* for 3.5 h. Untreated virus was centrifuged as a control. The gradients were analyzed on an ISCO model 640 density gradient fractionator using UA-5 absorbance monitor. The viral zones were collected, diluted fourfold with the buffer and centrifuged (90 000 × *g*, 2.5 h) to sediment the virus. The pellets of treated and untreated virus were resuspended and inoculated on the opposite halves of *N. glutinosa* leaves.

3. Results and discussion

Both flower and leaf extracts of *C. rupestris*, extracted with 45% ethanol and inoculated together with the virus to *N. glutinosa*, caused a significant reduction of local lesion number (IP = 80% and 90%, respectively). The purified flavonoid, identified as quercetagetin 3′-methylether, applied in the same way, exhibited even higher levels of antiviral activity (IP = 99%). On the other hand, extracts, either in 90% ethanol or ether, showed insignificant or no reduction, respectively. This was expected because flavonoids are mainly water soluble compounds (Harborne, 1984). In this experiment, and also in those described below, the applied concentrations of pure flavonoid or crude extracts did not cause any cytotoxic effect on the treated leaves.

The crude extracts or purified flavonoid preparations were also applied before virus inoculation, either by mechanical inoculation, or immersion of the root system (*N. megalosiphon*) or the leaf petioles (*N. glutinosa*) in the flavonoid solution. None of these pre-applications inhibited virus infection. This fact, together with other results presented in this paper, confirm the results of Verma (1973), French et al. (1991), and French and Towers (1992) which showed that the reduction of infectivity occurs only if virus and flavonoids are applied simultaneously.

The 45%-ethanol extracts, added to the virus inocula, strongly reduced the lesion number on inoculated leaves of the systemic host *N. megalosiphon*. Only 2–3 lesions in total appeared on three inoculated leaves of each plant in contrast with 90–120 lesions in the control. In spite of the local infection, 40% of infected plants did not produce any systemic symptom and the upper leaves remained virus-free until the end of the experiment (1 month, approximately). However, the plants which reacted systemically (60%) contained a similar virus concentration as the control plants inoculated with the virus only. It seems that the quoted absence of systemic infection resulted from a mutual effect of the flavonoid-inhibited primary infection and the diversity in susceptibility of particular plant specimens inoculated. Furthermore, the few necrotic lesions which appeared on inoculated

leaves of test plants reached the same size as the lesions on the control plants, indicating that the flavonoid did not inhibit symptom development.

Both leaf disc incubation test using *N. megalosiphon*, and the test in which the virus was inoculated to *N. glutinosa* before the flavonoid application, also failed to show any significant inhibition of virus infection (IP less than 5%, if any). This finding suggests that the flavonoid was ineffective in inhibiting the virus multiplication.

Further experiments were conducted to investigate whether the flavonoid has any direct virucidal effect, either by destabilizing or inactivating TBSV particles in the inocula. By density gradient centrifugation of the virus-flavonoid mixture (IP = 99%) a virus band was obtained. Comparison of the UV-absorption peak with that of the control showed a small difference in size, but no differences in shape and position of the bands. The results suggest that the flavonoid causes no damage to the TBSV virions. The separated flavonoid UV-absorption zone was present at the top of the gradient and the viral zone below again exhibited normal infectivity (IP = 1%). The amount of the flavonoid, eventually bound to the virus, may be so small that this cannot be measured by the techniques used.

Our findings are in agreement with those of French and Towers (1992) who stated that potato virus X (PVX) regained full infectivity following removal of unbound quercetin, concluding that quercetin has no direct virucidal effect on PVX particles. Both results can be explained by simultaneous inoculation of the flavonoid and the virus, and, as a result, the flavonoid subsequently occupies the sites at which the infection has to start and thus prevents infection. The mechanism of antiviral activity of the quercetagetin 3′-methylether and quercetin differs from that of 3,7,3′,4′-tetramethylquercetin (tetra-MQ) against tobacco mosaic virus (TMV) (French et al., 1991). According to the authors, incubation of TMV particles with tetra-MQ resulted in the formation of a stable virus-flavonoid complex. Recovery of the virions by ultracentrifugation and their infectivity were both reduced approximately by half.

The results presented in this paper (summarized in Table 1) indicate that the antiphytoviral activity of quercetagetin 3′-methylether against TBSV may not be the consequence of acquired resistance, inhibition of virus multiplication, symptom development suppression, or direct virus inactivation. These results are in agreement with the findings of French and Towers (1992) concerning the mechanism of antiphytoviral activity of quercetin against PVX. As inhibition failed to take place by application of flavonoid before and 30 s immediately after virus inoculation, the flavonoid may be interfering with an early event in virus-host interaction. French and Towers (1992) suggested that flavonoids inactivate recognition sites on the viral coat protein which interact with host recognition sequences required for the initiation of infection. The results of our investigations have shown that this proposed mode of flavonoid action may also be associated with the quercetagetin 3′-methylether–TBSV–*N. glutinosa*/*megalosiphon* system used in our experiments.

Table 1
The effect of the flavonoid from *Centaurea rupestris* L. on tomato bushy stunt virus (TBSV) infection (expressed as inhibition percentage, IP) in two *Nicotiana* hosts

Drug application to host plant	Effect on virus infection
Simultaneous inoculation to *N. glutinosa*	
crude 45%-ethanol leaf-extract	IP = 90%
crude 45%-ethanol flower-extract	IP = 80%
crude 90%-ethanol leaf- or flower-extract	Insignificant
crude ether leaf- or flower-extract	No inhibition
purified flavonoid from flowers	IP = 99%
Preinoculation* (1 h) to *N. glutinosa*	No inhibition
Preapplication** (2 days) by means of absorption by	
root system of *N. megalosiphon*	No inhibition
leaf petioles of *N. glutinosa*	No inhibition
Postinoculation* (30 s) to *N. glutinosa*	No inhibition
Postapplication*** by *N. megalosiphon* leaf disc incubation test	No inhibition

*Crude extracts or purified flavonoid.
**Crude 45%-ethanol extract of flowers.
***Purified flavonoid.

French et al. (1991) and French and Towers (1992) found that quercetin, by its direct contact with virus in vitro, strongly inhibits PVX but not TMV, and that certain *O*-methylated derivatives of quercetin are less effective inhibitors. French and Towers (1992) also found that quercetagetin (6-hydroxyquercetin) has no effect on PVX infection. However, our 3′-*O*-methylated quercetagetin was a strong inhibitor of TBSV. The results obtained by the above mentioned authors and the findings from our work suggest that the flavonoid efficiency depends on the combination of virus and specific *O*-methylated flavonoid but not on the host plant.

The results presented by French et al. (1991), French and Towers (1992) and those obtained in this study support the claim that some flavonoids not only have an inhibitory effect on animal viruses, but also possess antiphytoviral activity.

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