INHIBITION OF TOBACCO MOSAIC VIRUS INFECTION BY QUERCETIN AND VITEXIN

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Summary. – The flavonoids, quercetin and vitexin were proved to reduce lesion number in the local hosts *Datura stramonium* and *Chenopodium amaranticolor* infected with Tobacco mosaic virus (TMV). Both flavonoids also reduced the virus concentration in systemically infected tobacco plants. This effect was restricted to an early stage of infection and correlated with an induced synthesis of salicylic acid (SA) and kaempferol suggesting their possible defensive role in the infected plant tissue. Since the tested flavonoids did not bind to the virus particles, their antiphytoviral activity was probably not based on a direct virus inactivation.

Keywords: Tobacco mosaic virus; antiphytoviral activity; flavonoids; quercetin; vitexin

Introduction

Flavonoids are a broad class of more than 6,000 low-molecular-weight secondary metabolites that are present in all vascular plants (Harborne and Williams, 2000). Their structure is usually characterized by a C_6-C_3-C_6 carbon skeleton. These phenolic compounds afford protection against ultraviolet radiation, pathogens, and herbivores (Heim *et al*., 2002). They are also important for normal plant growth and development. Flavonoids act as the colored pigments of flowers but also as enzyme inhibitors and chelating agents of metal ions. They also affect photosensitization and energy transfer, respiration and photosynthesis levels, and the distribution of plant growth hormones and regulators (Wasson *et al*., 2006; Cushine and Lamb, 2005; Di Carlo *et al*., 1999).

Due to their interesting biological activities, flavonoids are important not only for plants, but also for animal organisms including humans. Increasingly, these compounds are becoming the subject of medical research because of their beneficial health effects, which are attributed mostly to their antioxidant and chelating abilities (Heim *et al*., 2002). A recent area of research that is of particular interest is their antiviral activity. A number of reports provides data about antiviral activities of flavonoids. Flavopiridol, a known cyclin-dependent kinase inhibitor, has been shown to have a potent *in vitro* activity against Human immunodeficiency virus 1, Herpes simplex virus 1 and 2, Human cytomegalovirus and Varicella-zoster virus (Schang, 2002). Furthermore, the release of replicated adenovirus genome from the nuclear matrix was shown to be tyrosine kinase-dependent. This important step in the viral life cycle was inhibited by known tyrosine kinase inhibitors, such as the flavonoids genestein and quercetin (Angeletti and Engler, 1996). However, only a few studies have revealed the antiphytoviral activity of flavonoids (Rusak *et al*., 1997, 2007; Malhotra *et al*., 1996; French and Towers, 1992; French *et al*., 1991; Verma, 1973). The efficiency of plant 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 (Rusak *et al*., 1997, 2007).

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Abbreviations: BSA = bovine serum albumin; DMSO = dimethyl sulfoxide; HPLC = High performance liquid chromatography; p.i. = post inoculation; PVX = Potato virus X; SA = salicylic acid; TMV = Tobacco mosaic virus

In our study, various flavonoid compounds were preliminary investigated to demonstrate their inhibitory effect on TMV infection in some local host plants. Two of them, quercetin and vitexin revealed a significant reduction of local lesion number. Consequently, the antiphytoviral activity of these compounds representing different classes of flavonoids (flavonol and C-glycosylflavon, respectively) was the subject of several more detailed studies. Quercetin, one of the most abundant flavonoids in plants has been shown to possess a broad spectrum of biological activities. On the other hand, the biological activities of vitexin are almost unknown and only a few articles dealing with its biological effects have been published so far (Hajdu *et al*., 2007; Kim *et al*., 2005a,b; Li *et al*., 2002). A recently reported very sensitive and fast method was used to test the binding capacity of flavonoids to TMV nucleoprotein (Gutzeit *et al*., 2004). The method is based on the fact that weak fluorescence of flavonoids is strongly enhanced upon binding to specific proteins.

The aim of the present study was to contribute to the knowledge of antiphytoviral activity of flavonoids by providing data about some other flavonoids, flavonoid-virus, or flavonoid-virus-host combination, as well as some new methodological approaches in this field.

Materials and Methods

Virus and host. TMV was propagated in *Nicotiana tabacum* L. cv. Samsun and purified from systemically infected leaves by a standard method (Gibbs and Harrison, 1976). The purified virus preparation was stored at -20°C and diluted with inoculation buffer (0.06 mol/l phosphate buffer, pH 7.0) and inoculated to the local host *Chenopodium amaranticolor* to yield 20–30 lesions per leaf. The inoculum prepared in this way was used to inoculate also *Ch. murale* L. and *Datura stramonium* L. as the local hosts, and *N. tabacum* L. cv. Samsun as a systemic host.

Flavonoids. The flavonoids quercetin, myricetin, apigenin, naringenin (Sigma), and vitexin (Fluka) were dissolved in dimethyl sulfoxide (DMSO) to give the concentration 250 mmol/l. The stock solutions were stored at 4°C. The final concentration of the flavonoid in virus inoculum was adjusted to 250 µmol/l.

Application to the local host plants. Antiphytoviral activity of the flavonoids against TMV was determined by the half-leaf method. One half of the leaf was inoculated with the virus preparation containing 250 µmol/l of a flavonoid and the opposite half of the same leaf was inoculated with the same virus concentration without the flavonoid. All treatments were repeated three times on plants selected for uniformity and grown in a greenhouse (23°C; 16:8 h light/dark cycle). The local lesions were counted 7 days post inoculation (p.i.) and the inhibition percentage was calculated by comparing the number of viral lesions on the two leaf halves according to the formula 1:

$$IP = \frac{CK - A}{CK} \text{ x } 100,$$

where IP = antiviral inhibition in %, CK = average number of viral lesions on the control leaf half, A = average number of viral lesions on the flavonoid-treated leaf half.

Application to the systemic host plants. To determine the antiphytoviral activity of quercetin and vitexin, the virus concentration in flavonoid-treated and control systemically infected plants was measured and compared. In this experiment, four groups of *N. tabacum* plants (cca 100 plants per group) were inoculated (two leaves on each plant) with: (a) a mixture of the virus inoculum containing 250 µmol/l flavonoid; (b) a virus inoculum without flavonoid (control); (c) a virus inoculum containing 250 µmol/l flavonoid, followed by daily treatment of the upper leaves with the inoculation buffer containing 250 µmol/l of flavonoid for consecutive 14 days; (d) the virus inoculum followed by daily treatment with inoculation buffer only for consecutive 14 days (control). The leaves of all plant groups were collected at the day 3, 5, 8, 11, and 14 p.i., and the virus concentration was determined by the spectrophotometer BECKMAN DU 530 in a purified preparation according to the formula 2 (Gibbs, 1977):

$$c = \frac{A_{260}}{3} \text{ x R},$$

where c = virus concentration, A_{260} = absorption ($\lambda = 260$ nm), R = dilution. The inhibition percentage was calculated by comparing the virus concentration in the flavonoid-treated and control groups according to the formula 3:

$$IP = \frac{CK - A}{CK} \text{ x } 100,$$

where IP = viral inhibition in %, CK = virus concentration in the control group, and A = virus concentration in the flavonoid-treated group.

dsRNA was isolated from TMV-infected leaves of *N. tabacum* collected at day 5, 10, 15, and 20 p.i. according to the procedure of Kearney *et al*. (1990), modified by Škorić *et al*. (2000). dsRNA was separated by electrophoresis in 1.2% agarose gel and stained with ethidium bromide. Besides dsRNA, the virus concentration in the same plant tissue was measured to find out the correlation between dsRNA band intensity and virus particle concentration.

Quantification of fluorescence. The fluorescence elicited by the TMV-flavonoid interaction was determined in black 96-well microtiter plates (Nunc) at $\lambda = 520$ nm (excitation at $\lambda = 485$ nm). The reaction mixture (100 µl/well) in the phosphate buffer solution (pH 7.5) containing 0.1% DMSO, 2 mg/ml of the purified virus particles, and 40 or 250 µmol/l of the flavonoid, was incubated for 30 mins at room temperature before the fluorescence was quantified in a Microtiterplate Reader (FLUOstar OPTIMA, BMG). The fluorescence intensity was compared to that obtained in a control experiment with the flavonoid alone and TMV alone. In a parallel experiment, bovine serum albumin (BSA) was used as the standard protein and the fluorescence elicited by BSA-flavonoid interaction was quantified in the same way. For each combination of reactants, 3 experiments with 5 determinations were made.

High performance liquid chromatography (HPLC) analysis of flavonoids in the infected tobacco plants. The leaves of the systemic host *N. tabacum* were collected 3, 5, and 8 days p.i. from the 3 experimental groups of plants, simultaneously treated with vitexin or quercetin in mixture with the virus or quercetin was

continuously introduced for 8 days after virus/flavonoid inoculation. Plants inoculated with the virus without flavonoids, plants inoculated with the virus and continuously rubbed with phosphate buffer, and healthy untreated plants were used as 3 control groups. The collected leaves were washed once with DMSO diluted with redistilled water in a ratio 1:15 and twice with redistilled water to remove the exogenous flavonoid from the leaf surface. Lyophylized and powdered leaves (200 mg) were extracted with 70% methanol (1 ml) overnight (cca 20 hrs). After centrifugation (20 mins, 11,000 rpm) the supernatant was removed and stored at -20°C (unhydrolyzed extracts). Aliquots of 200 µl were hydrolyzed with 2 mol/l HCl (800 µl) in a boiling water bath for 30 mins. After triple extraction with diethyl-ether (800, 800, and 600 µl) followed by vaporization, the samples were resuspended in 96% ethanol and stored at -20°C (hydrolyzed extracts).

HPLC system (Agilent 1100 Series) equipped with a quaternary pump, multiwavelength UV/VIS detector, autosampler, and fraction collector was used for the detection and quantification of flavonoids in prepared samples. The column (5 µm Zorbax RX-C18), sized 250 x 4.6 mm (Agilent Technologies) had the injection volume of 50 µl and the flow rate of 1.0 ml/min at 35°C. The elution buffer consisted of the solvent A (5% formic acid) and solvent B (acetonitrile). A linear gradient from 10 to 56.4% B within 29 mins, followed by the gradient from 56.4 to 100% B in the next 6 mins was applied and absorbance was taken at $\lambda = 268$, 280, 310, 350, and 374 nm.

Fig. 1

Reduction of TMV local lesion number on the flavonoid-treated (right) and untreated (left) leaf half of *Ch. amaranticolor* (a) and *D. stramonium* (b)

Results and Discussion

Vitexin applied to *Ch. amaranticolor* and quercetin applied to *D. stramonium* caused significant reduction of the lesion number in TMV-infected plants with an antiviral activity rate of 30 and 26%, respectively (Fig. 1). A slightly lower efficiency was recorded for naringenin with a rate of 15% on *Ch. amaranticolor*. The two other flavonoids tested in the preliminary investigation (apigenin, myricetin) proved to be only slightly efficient or not efficient at all.

Quercetin was previously reported by Verma (1973) as an inhibitor of Potato virus X (PVX), and the mechanism of local-lesion reduction using *Ch. quinoa* was based on the interference with the viral coat protein – host interaction as proposed by French and Towers (1992). The same early event was influenced by this flavonoid in the Tomato ringspot virus cycle, as explained by Malhotra *et al*. (1996) about the antiphytoviral activity in the local host *Ch. quinoa*. In contrast to results obtained in *Ch. amaranticolor*, pretreatment of *Ch. quinoa* with quercetin failed to inhibit the infectivity of PVX (French and Towers, 1992; Verma, 1973). Furthermore, quercetin did not affect TMV local lesion number in *N. glutinosa* (French *et al*., 1991). The same authors concluded that the methylation of flavons at the 3'-position and either the 4'- or 7'-position is required for the inhibition of TMV. Results obtained in our most recent and also the previous study as well as the results mentioned above indicated that the rate and mode of virus inhibition depended on the flavonoid-virus-host combination studied (Rusak *et al*., 1997). For this reason, the inhibitory effect of each flavonoid was tested in our investigation on 3 different local-host species.

Replication of the viral single-stranded RNA genome generates a sufficient quantity of dsRNA. This stable replicative form can be isolated from a small amount of infected tissue and recognized by a standard electrophoretic analysis. Although the use of dsRNA is unusual as a measure of virus multiplication, recently published results obtained with Cucumber mosaic virus satellite RNA demonstrated at least a rough correlation between these two parameters (Rusak *et al*., 2007). However, the correlation between dsRNA band intensity and the virus particles concentration was not confirmed in our experiments (Table 1, Fig. 2). Consequently, dsRNA patterns were not used for assessment of flavonoid influence on the virus multiplication process.

So far, most experiments have assessed antiviral activity of the examined substances using local host plants and half-leaf rubbing method (Xia *et al*., 2006; An *et al*., 2001; Sano, 1999; Rusak *et al*., 1997). Some papers also report about virus multiplication measured by ELISA in flavonoid solution floating leaf discs from systemically infected hosts (Rusak *et al*., 1997; French and Towers, 1992; French *et al*., 1991). To

Table 1. TMV concentration in virus preparations purified from systemically infected plants *N. tabacum* 5, 10, 15, and 20 days p.i.

Day (p.i.)	A_{260}	A_{280}	A_{260}/A_{280}	Virus concentration (mg/ml)
5	1.30	1.95	1.50	4.3
10	2.75	1.96	1.41	9.2
15	2.08	1.50	1.38	6.9
20	1.35	1.04	1.29	4.5

assess the flavonoid antiphytoviral effect in a systemically infected host, we compared virus concentration in the flavonoid-treated and untreated tobacco plants continuously during 14 days p.i. Vitexin and quercetin were selected for those experiments because of their promising level of antiviral activity in local host plants. The virus concentration was determined in preparations purified from systemically infected leaves collected 3, 5, 8, 11, and 14 days p.i. to provide complete information on viral dynamics possibly influenced by the flavonoid, especially at the early stage of infection.

With the exception of very early stages of infection (until the day 3 p.i.), virus concentration was significantly reduced by vitexin introduced only once together with the virus. The most intensive antiviral effect was recorded 8 days p.i., when the virus concentration was reduced by 40% with a decreasing tendency in later stages of infection (Fig. 3). When applied continuously, vitexin was inefficient as a virus inhibitor. Surprisingly, increased concentration of the virus was established in treated plants 5 and 8 days p.i. (Fig. 3). This fact could be explained by cytotoxic effect of vitexin on host plants by its prolonged application. The virus replication induced by vitexin on day 3 p.i. in one-time treatment could also be explained by the same cytotoxic effect of vitexin on young plants.

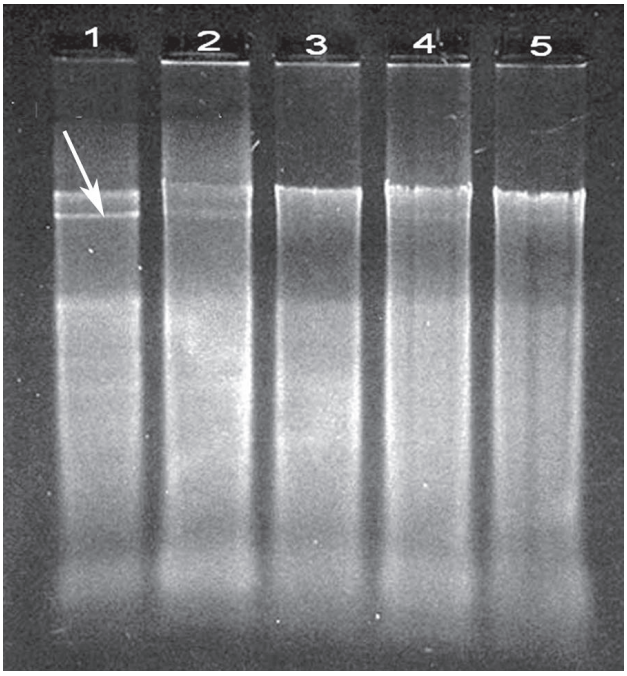

Fig. 2

Electrophoresis of dsRNA (arrow) isolated from TMV-infected leaves of *N. tabacum* 5, 10, 15, and 20 days p.i. (lane 1, 2, 4, and 5, respectively)

Non-infected leaves (lane 3).

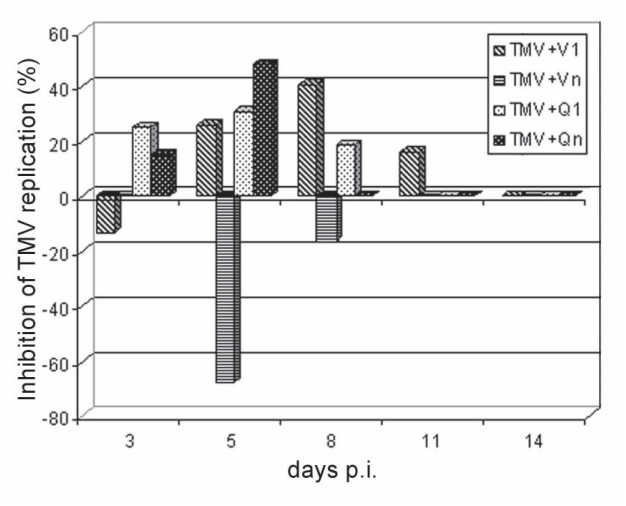

Fig. 3

Inhibition (%) of TMV concentration in infected *N. tabacum* following single (1) or continuous (n) treatment with vitexin (V) or quercetin (Q) related to TMV concentration in untreated infected plants

On the other hand, the inhibition of TMV replication was demonstrated for quercetin applied in both ways: simultaneously with the infecting virus in single treatment or continuous treatment during the systemic virus infection. In the single quercetin-treatment, the virus concentration was reduced on the days 3, 5, and 8 p.i. with 25, 31, and 19% inhibition, respectively. Later on, the antiviral activity was not detected. Similarly, the antiphytoviral effect of the continuous quercetin-treatment was detected only on day 3 and 5 p.i. with the inhibition rate of 15 and 48%, respectively (Fig. 3). It is generally observed that TMV concentration in systemically infected tobacco plants reaches its plateau 11 to 14 days p.i. TMV probably takes that much time to invade tobacco plant completely and fulfill the multiplication process in each cell of the systemically infected tissue. Obviously, the antiviral effect was restricted to this period regardless of the flavonoid tested and the approach used in the experiment. Again, this suggested that a flavonoid acted by interfering with the early stage of infection of the individual cells in either local or systemic infection.

Since the usually weak florescence of a flavonoid is strongly enhanced upon binding to a specific protein (Gutzeit *et al.*, 2004),

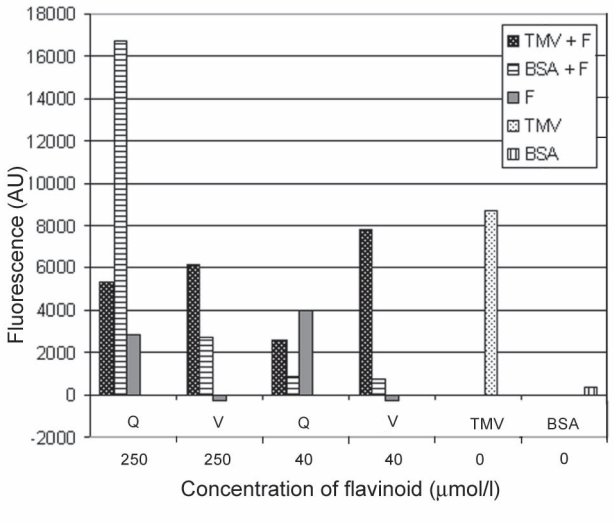

Fig. 4

Fluorescence of TMV and BSA (control) examined at λ = 520 nm after incubation with flavonoids (TMV+F, BSA+F)

Two concentrations 250 and 40 μmol/l of quercetin (Q) and vitexin (V) were used. The fluorescence was expressed in absorbance units (AU).

Table 2. Absorbance (mAU) of salicylic acid and flavonoids kaempferol and quercetin in hydrolyzed and unhydrolyzed extracts of TMV-infected leaves of *N. tabacum* treated with the flavonoids quercetin or vitexin simultaneously

Day (p.i.)	Sample	mAU		
		Salicylic acid (unhydrolyzed)	Quercetin (hydrolyzed)	Kaempferol (hydrolyzed)
3	TMV + Q	4	17	51
	TMV + V	6	ND	ND
	TMV	5	Traces	2.5
	Control	6	ND	3.7
5	TMV + Q	4	ND	Traces
	TMV + V	9	ND	ND
	TMV	5	ND	Traces
	Control	4	2	6
8	TMV + Q	10	ND	3.2
	TMV + V	11	ND	4.6
	TMV	9	1.5	7.3
	Control	9	3.5	17

TMV + Q = infected and treated with quercetin; TMV + V = infected and treated with vitexin; TMV = infected, untreated; control = uninfected, untreated; ND = not detected;

possible changes in the fluorescence intensity were investigated to demonstrate the capacity of the flavonoids to bind with TMV. Neither quercetin nor vitexin induced fluorescence changes following incubation with the purified virus preparation (Fig. 4). Since the flavonoids tested did not bind with TMV particles, their direct virucidal effect was not presumable.

HPLC analysis of unhydrolyzed methanolic extracts of tobacco plants simultaneously inoculated with TMV and vitexin revealed an increased absorbance of SA, indicating its increased concentration, on day 5 p.i. However, on day 3 p.i. the concentration of SA was approximately the same as in the untreated TMV-infected plants (Table 2). This correlates with the antiviral effect established on the day 5 but not day 3 p.i. with simultaneous inoculation of the virus and vitexin.

The increased SA concentration recorded at day 3 p.i. in tobacco plants continuously treated with quercetin (Table 3) fitted well with the reduced virus concentration at that stage of infection or preceded the maximal antiviral effect recorded later. These results revealed the possibility that the antiviral effect of flavonoids is mediated by the induction of SA synthesis and probably based on some process related to systemic acquired resistance.

When hydrolyzed methanolic extracts were analyzed by HPLC, an increased concentration of kaempferol and quercetin was established 3 days p.i. for quercetin-treated tobacco tissue in both simultaneous application and continuous treatment (Table 2, 3). This increased synthesis of kaempferol correlated with the decreased virus concentration in the quercetin-treated infected tissue. Kaempferol and quercetin are closely related in their biosynthetic pathways and it could be supposed that external application of quercetin could have some implications on the biosynthesis of flavonoids that are structurally related to quercetin. Furthermore, higher concentration of phenolics in the infected plants could have some implications on the defense response in these plants, since the involvement of these compounds in plant-pathogen interaction is well known (Cushine and Lamb, 2005; Dixon *et al.*, 2002; Berhow and Vaughn, 1999).

Our previous investigations as well as the investigations carried out in this study confirmed that flavonoids might

Table 3. Absorbance (mAU) of salicylic acid and flavonoids kaempferol and quercetin in hydrolyzed and unhydrolyzed extracts of TMV-infected leaves of *N. tabacum* treated with quercetin continuously

Day (p.i.)	Sample	mAU		
		Salicylic acid (unhydrolyzed)	Quercetin (hydrolyzed)	Kaempferol (hydrolyzed)
3	TMV + Q	80	14	45
	TMV	59	ND	8
	Control	40	ND	4
5	TMV + Q	58	Traces	17
	TMV	62	8	35
	Control	64	ND	7.5
8	TMV + Q	52	4.5	30
	TMV	37	ND	4.5
	Control	60	4	21

For legend see Table 2.

interfere with the early event of the plant virus infection (Rusak *et al*., 1997). Based on a limited number of publications published so far in this field (Rusak *et al*., 1997, 2007; Malhotra *et al*., 1996; French and Towers, 1992; French *et al*., 1991), it could be concluded that the mode of virus inhibition depended on the flavonoid-virus-host combination studied and that there was no universal mode of antiphytoviral action of flavonoids. This paper presented some new data about the flavonoid antiphytoviral effect and tried to clarify its possible mode of action. However, some new questions emerged that should be solved. This is the first report about the induction of kaempferol and SA synthesis by exogenously applied flavonoids that correlates with the decreased virus concentration in the infected plants. This induction suggested synergistic antiphytoviral action of exogenously applied flavonoids and endogenously synthesized phenolics. Further investigation about the antiphytoviral mechanisms of flavonoids could lead to a possible utilization of these compounds in a control of plant virus diseases.

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