Phyton (Austria) Special issue: "Plant Physiology"	**Vol. 39**	Fasc. 3	(259)-(264)	30. 11. 1999

Accumulation Rate of Rutin is Decreased after Infection of Susceptible Potato Cultivar with PVY^{NTN}

By

Samo KREFT[1,4)], Maruša POMPE[2)], Maja RAVNIKAR[2)], Andrej UMEK[1)] & Borut ŠTRUKELJ[1,3)]

K e y w o r d s : Potato virus Y^{NTN}, *Solanum tuberosum*, rutin, HPLC, capillary electrophoresis.

S u m m a r y

KREFT S., POMPE M., RAVNIKAR M., UMEK A. & ŠTRUKELJ B. 1999. Accumulation rate of rutin is decreased after infection of susceptible potato cultivar with PVY^{NTN}. - Phyton (Horn, Austria) 39 (3): (259) - (264).

Potato virus Y^{NTN} is a member of a Potyviridae family, which causes a great damage in the cultivation of potato (*Solanum tuberosum*). Many cultivars (for instance cv. Igor) are sensitive to the infection and some are resistant or tolerant (for instance cv. Sante and cv. Desirée, respectively). In our study we followed the changes in secondary metabolite profile after infection of potato plants of the three cultivars. The plants were harvested 4 hours, 4 days and 14 days after infection. They were freeze- dried and extracted with methanol. The extracts were analysed by HPLC. By comparison of chromatograms of infected and control plants, we found only one peak, which differed significantly. The corresponding substance was later isolated and identified as rutin.

The rutin content in the plants of all cultivars increases during the early development stage. The infection with PVY^{NTN} inhibits this accumulation in susceptible potato cultivar (cv. Igor) by more than 50 %, but it has no significant influence on the rutin accumulation in cv. Sante and cv. Desirée. There are two possible explanations for the observed phenomenon. Either the rutin has an active role in defence mechanism of potato plant, or its decreased accumulation is only due to redirection of biosynthetic pathways from rutin synthesis to the synthesis of other phenolic substances. To test this possibilities we grew infected plants in vitro in the medium containing

[1)] Faculty of Pharmacy, University of Ljubljana, Aškerčeva 7, SI-1000 Ljubljana, Slovenia.

[2)] Laboratory for Plant Physiology, National Institute of Biology, Večna pot 111, SI-1000 Ljubljana, Slovenia.

[3)] Department of Biochemistry and Mol. Biology, Jozef Stefan Institute, Jamova 39, SI - 1000 Ljubljana, Slovenia.

[4)] to whom correspondence should be addressed. E-mail: samo.kreft@ffa.uni-lj.si

different concentrations of rutin (or quercetin). Since concentration of viruses did not differ significantly among the plants growing in the presence or in the absence of rutin (or quercetin), we assume that rutin has no direct role in virus multiplication.

Introduction

Many physiological and phytochemical changes are caused by biotic stress (bacteria, fungi, viruses, herbivores) and abiotic stress (UV radiation, drought, extreme temperatures, mechanical damage). The response of the plant to the stress is well described at different levels: morphology of the plant - lesions, necroses (PENNAZIO 1995), etc.; hormones (TIZIO 1996); signal molecules (BOWLES & al. 1994); secondary metabolism, for example phytoalexins; proteins as pathogenesis related proteins; mRNA or gene expression level (SEO & al. 1997). But there are only few reports on the changes in secondary metabolism after virus infection (STINTZI & al. 1993). In this study we followed the changes in secondary metabolites content in different potato cultivars after infection with PVY^{NTN}. Some potato cultivars for instance cv. Igor are especially sensitive for the viral infection. The virus induces the appearance of severe disease symptoms, severe mosaics on the leaves of infected plant and ring-shaped necroses on the tubers around the eyes (LE ROMANCER & al. 1994). Some potato cultivars as cv. Desirée are more tolerant. The plants are infected, but they do not develop a severe signs of disease. Finally, some cultivars as cv. Sante are resistant to the infection.

In an attempt to investigate the relationship between in vivo viral infection and secondary metabolite contents, we performed two sets of experiments: one to test the influence of viral infection on secondary metabolism and the other to test the influence of secondary metabolites on viral multiplication.

Materials and Methods

Plant material

The three cultivars (Igor, Desirée and Sante) of potato *Solanum tuberosum* L., were grown in pots in growing chambers (Köttermann KG 2730) at constant temperature 20 ± 1 °C, humidity 70 - 80 % and illumination 5.5 -10.1 W/m^2 12h per day. All plants were micropropagated using stem node culture. 4 weeks after transplantation to soil one third of the plants was leaf inoculated by sap of PVY^{NTN} infected plants, one third of the plants was inoculated by sap of healthy plants, and the last third of the plants was not treated. The plants were harvested 4 hours, 4 days and 14 days after infection. The plants were immediately frozen in a liquid nitrogen and kept at -70°C. The infection of the plants was checked 14 days after infection using ELISA test.

HPLC analysis

The frozen samples were freeze-dried and finely powdered. The 50 mg aliquot of the sample was extracted with 10 ml of methanol under the reflux at 60°C for 10 min followed by 5 min sonification. The extract was centrifuged for 5 min at 6.000 g and the supernatant filtered (Sartorius Minisart NML 0.2 µm). The sample was then analysed by HPLC to see how the potato plants respond to the viral infection (column: nucleosil 100 C-18, 250 x 4.6 mm; solvent: MeOH-H_2O, linear gradient from 35 % to 65 % MeOH in 2^{nd} to 11^{th} minute; flow rate: 1 ml/min; temperature: 25°C; detection 254 nm; injection volume: 50 µl).

Identification of the responding substance

The green parts of potato (cv. Sante) plants (3 kg of fresh weight) were two times sequentially macerated for 24 hours with 4 l of methanol. The extracts were filtered and methanol was evaporated under reduced pressure. The residue was mixed with 1 l of distilled water and sequentially extracted with diethylether, ethylacetate and n-buthanol (3 x 300 ml of each solvent). The HPLC analysis of all the fractions showed, that the responding substance was in n-buthanol fraction. The solvent from this fraction was evaporated, the residue dissolved in 30 ml of methanol and applied on a column (850 x 35 mm) filled with Sephadex LH-20. The column was eluted with ethanol and the collected fractions (10 ml) were analysed by HPLC. The fractions that contained the responding substance were combined and applied once more to the same column. The isolated substance was analysed by UV/VIS spectroscopy, mass spectroscopy and NMR and identified as rutin.

Capillary electrophoresis

The absolute concentration of rutin was determined on a Hewlett Packard HP 3D Capillary Electrophoresis System. Bare fused-silica capillaries (inner diameter 50 μm, length 57 cm, with bubble cell) were used. The buffer was: 50 mM borate,100 mM SDS (pH 9.3). The samples were injected by application of pressure (20 mbar for 20 seconds). The detection was carried out at 380 nm.

Antiviral activity test

Rutin and quercetin were dissolved in DMSO and added in different concentrations (0, 1, 100 and 500 μM) to the MS medium on which stem nodes of PVY^{NTN} infected and healthy potato plants (*Solanum tuberosum* L. cv. Pentland squire) were planted. This cultivar was chosen because its growth in tissue culture is effected more severely by infection with PVY^{NTN} than the cultivar Igor. With the increase of rutin or quercetin concentration in the media also DMSO concentration in the media increased, what might slightly modify the results at 100 and 500 μM of rutin and quercetin, while at 1 μM of rutin the DMSO concentration was under 0.1%, which was estimated as concentration that has no effect on plants (LE ROMANCER & al. 1994). The number of roots, stem nodes and lateral shoots were counted and the length of the shoots and the concentration of the virus were measured after 4 and/or 7 weeks. DAS ELISA and monoclonal antibodies against necrotic strain of PVY (Bioreba, Switzerland), and standard dillutions of purified PVY^{NTN} on each microplate were used to determine virus concentration in each plantlet.

Results

The effect of viral infection on the secondary metabolites

The content of approximately 20 substances was determined in methanolic extracts. Among them only one substance differed in the extracts from infected and control plants. The corresponding peak was identified as rutin. The concentration of rutin increased 3.4 fold, 5.8 fold and 2 fold in the control plants of cultivars Igor, Sante and Desirée, respectively, during the 14 days of investigation (Fig. 1). We observed approximately two fold lower levels of rutin in infected plants of cv. Igor, compared to control plants, but the concentration was also increasing during the cultivation. Cultivar Igor is the most susceptible among tested cultivars. The infection did not affect the rutin content in other two cultivars very much. There was only a slight increase in cv. Sante and a small decrease in cv. Desirée. The two

sets of control plants: healthy sap inoculated and not treated plants did not differ significantly.

The absolute concentration of rutin in 2 months old potato plants (cv. Igor, and cv. Sante) determined by capillary electrophoresis was 3.2 µg/g d.w. and 1.6 µg/g d.w., respectively.

The effect of rutin and quercetin on the propagation of virus

No significant effect of rutin and quercetin on the viral concentration was observed in the plants growing in the medium containing both flavonoids (Fig. 2).

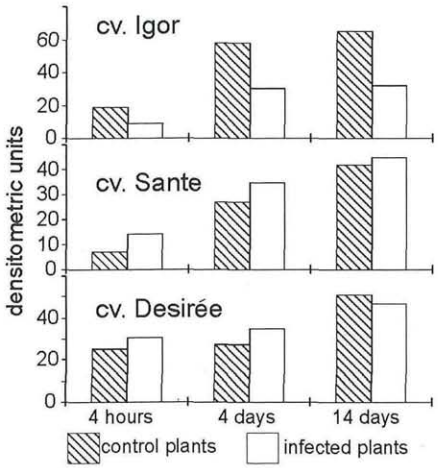

Fig. 1. Rutin content in control and infected plants of the three cultivars 4 hours, 4 days and 14 days after infection.

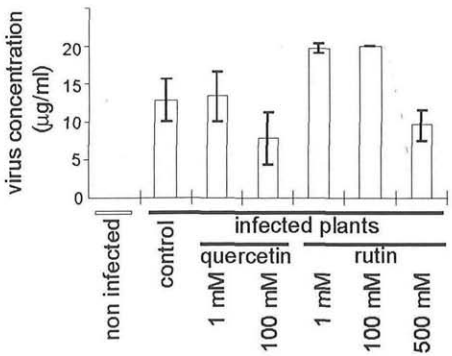

Fig. 2. Viral concentration in healthy and PVY^{NTN} infected plants grown on control medium and media supplemented with rutin or quercetin. Standard errors are indicated by error bars.

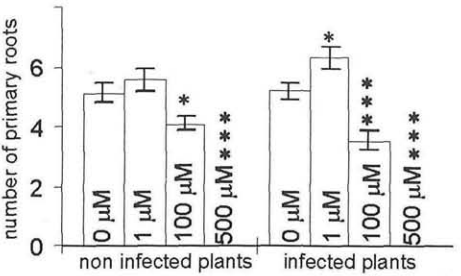

Fig. 3. Number of primary roots of healthy and PVY^{NTN} infected plants grown on media containing 0, 1, 100 or 500µM of quercetin after 4 weeks. The values indicated with stars are significantly different than the value at 0 µM quercetin (*: $p<0.05$, ***: $p<0.001$). Standard errors are indicated by error bars.

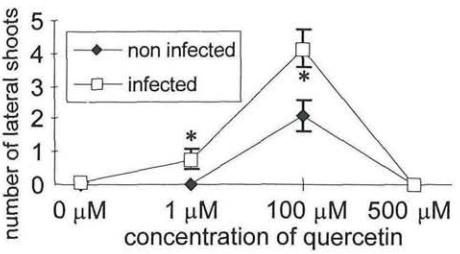

Fig. 4. Number of lateral shoots of healthy and PVY^{NTN} infected plants grown on media containing 0, 1, 100 or 500µM of quercetin after 8 weeks. At values indicated with star, the infected plants significantly differ from the non infected plants ($p<0.05$). Standard errors are indicated by error bars.

The effect of rutin and quercetin on the plant morphology

Rutin and quercetin influenced on the morphology of healthy and PVY^{NTN} infected plants generally in the same way, although there were some differences in a few growth parameters. High concentrations (100µM) of rutin and quercetin inhibited the growth of shoots and the development of roots, but significantly stimulated the development of lateral shoots (Fig. 4). Under the influence of low concentration (1µM) of rutin and quercetin the number of primary roots increased (Fig. 3). In some experiments we even noticed, that PVY^{NTN} infected plants on the media supplemented with 1µM of rutin or quercetin reached the same height as healthy plants on control media.

Discussion

The effect of quercetin and some other flavonoids on potato virus X (PVX), tobacco mosaic virus (TMV) and tomato ringspot virus (TomRSV) was previously described (FRENCH & al.1991, FRENCH & TOWERS 1992, MALHOTRA

1996), but there are no reports about the content of this metabolites in infected plants. It was proposed, that quercetin inhibits an early event in the virus life cycle, but it does not influences the multiplication of the virus (MALHOTRA 1996). This is in accordance with our observations, that the rutin and quercetin do not reduce the virus concentration, when the infected plants of cv. Pentland squire are grown on the medium containing this flavonoids. All morphology and virus concentration studies were done in vitro on secondary infected plants of cv. Pentland squire, however we know that potato plant metabolism changes significantly when plants are grown in tissue culture.

Our study indicates that rutin and quercetin metabolisms of potato cv. Igor change during primary infection with PVY^{NTN}, therefore it would be interesting to estimate the influence of rutin and quercetin on virus infectivity and concentration during primary infection in vivo. Regarding the changes of endogenous rutin in susceptible cultivar Igor after infection and observation that 1μM of rutin and quercetin stimulate growth of infected shoots of cv. Pentland squire in vitro we could conclude that rutin might have important role in defence response of potato on PVY^{NTN} infection.

Acknowledgement

This project was supported by Ministry of Science and Technology of the Republic of Slovenia and by INCO-Copernicus Grant ERBIC 15CT960921. We would like to thank I. MAVRIČ for performing quantitative ELISA.

References

BOWLES D. J., GILMARTIN P. M., KNOX J. P. & LUNT G. G. 1994. Molecular botany: Signals and the environment. The biochemical society symposium 60. - The biochemical society, London.

FRENCH C.J. & TOWERS G.H.N. 1992. Inhibition of potato virus X by flavonoids. - Phytochemistry 31: 3017-3021.

— , ELDER M., LEGGETT F., IBRAHIM R.K. & TOWERS G.H.N. 1991. Flavonoids inhibit infectivity of tobacco mosaic virus. - Canadian Journal of Plant Pathology 13: 1-6.

LE ROMANCER M., KERLAN C. & NADELLECI M. 1994. Biological characterisation of various geographical isolates of potato virus Y inducing superficial necrosis on potato tuber. - Plant Pathology 43: 138-144.

MALHOTRA B., ONYYLAGHA J. C., BOHM B. A., TOWERS G. N. H., JAMES D., HARBORNE J. B. & FRENCH C. J. 1996. Inhibition of tomato ringspot virus by flavonoids. - Phytochemisty 43: 1271-1276.

PENNAZIO S. 1995. The hypersensitive reaction of higher plants to viruses: A molecular approach. - Microbiologica 18: 229-240.

SEO S., SANO H. & OHASHI Y. 1997. Jasmonic acid in wound signal transduction pathways. - Physiologia Plantarum 101: 740-745.

STINTZI A., HEITZ T., PRASAD V., WEIDWMANN-MERDINNOGLU S., KAUFFMANN S., GEOFFROY P., LEGRAND M. & FRITIG B. 1993. Plant 'pathogenesis-related' proteins and their role in defence against pathogens. - Biochemie 75(8): 687-706.

TIZIO R. 1996. Jasmonic acid and their derivates as plant growth regulators. - Biocell 20(1): 1-10.