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Glucose-6-phosphate dehydrogenase, ribonucleases and esterases upon tobacco mosaic virus infection and benzothiodiazole treatment in tobacco

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Abstract

Effect of the benzothiodiazole (BTH) pre-treatment was monitored during the acute infection stage in the susceptible and the hypersensitive tobacco plants infected with the tobacco mosaic virus (TMV). Dynamic changes in the contents of chlorophyll, the total proteins, and the pathogenesis related proteins (PR-proteins), and activities of ribonucleases (RNase), phosphomonoesterase (PME), phosphodiesterase (PDE), and glucose-6-phosphate dehydrogenase (G6P DH) were studied. Neither the protein nor the chlorophyll contents were significantly changed by the TMV infection and/or the BTH treatment. The BTH pre-treatment caused a substantial reduction in the multiplication of TMV in the locally-infected leaves of the hypersensitive cultivar Xanthi-nc (to 15.1 %). A lesser decrease (to 50.3 %) was observed in the locally-infected leaves of susceptible cultivar Samsun. But in the systemically-infected leaves of this cultivar, only a 4-d delay in the multiplication of TMV was found. In the locally-infected leaves of both cultivars, the activities of the RNase, PME, PDE and G6P DH were sharply increased during the acute phase of TMV multiplication (when compared with the healthy plants) and the curves of these activities correlated with the multiplication curves of TMV. The BTH alone also strongly enhanced the activities of these enzymes early after application. Only low additional increases in some enzymes and even slight declines in the others were observed when the inoculation of leaves of cultivar Xanthi-nc followed the pre-treatment with the BTH. No inhibition of the enzymes was observed when the direct effect of different concentration of the BTH (1 - 1000 μM) was examined *in vitro* during a measurement of the activity. The analysis of intercellular proteins by PAGE under native conditions shows the similar spectrum of the proteins extracted from either the BTH-treated or the TMV-infected tobacco cv. Xanthi-nc.

Additional key words: chlorophyll, *Nicotiana tabacum*, PAGE, ICF, protein, resistance, rRNA, virus RNA.

Introduction

Resistance can be induced locally (LAR) after contact with viruses that cause necrotic reactions (biotic induction) or systemically in untreated parts of plants when it is called systemic acquired resistance (SAR) (Ryals *et al.* 1996). This term was introduced by Ross (1961) to describe induction of resistance in tobacco against a range of viruses by prior inoculation with the necrosis-causing tobacco mosaic virus. Subsequently, induction of systemic resistance by plant pathogens has been described in over 100 host-pathogen systems (for review, see Sequeira 1983). Although this has not been generally tested, SAR seems to be a specific in terms of the range of pathogens against which the plant is protected (Kuć 1982). It is often assumed that the inducing organism should cause necrosis in order to induce SAR (Ward *et al.* 1991), although Roberts (1982) demonstrated that this is not a prerequisite.

Chemical agents that do not cause any visible symptoms have also been shown to induce resistance. White (1979) was able to induce resistance in tobacco against tobacco mosaic virus by injecting acetylsalicylic acid into the leaves. Since then, resistance induced

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Abbreviations: BTH - benzothiodiazole; dpBTH - day post BTH application; dpTMV - day post TMV inoculation; G6P DH - glucose-6-phosphate dehydrogenase; ICF - intercellular fluid; PDE - phosphodiesterase; PME - phospho-monoesterase; PR-proteins - pathogenesis related proteins; RNase - ribonuclease; SAR - systemic acquired resistance; TMV - tobacco mosaic virus.

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by salicylic acid, isonicotinic acid and benzo-(1,2,3)-thiodiazole-7-carbothioic acid-S-methyl ester (BTH) was demonstrated in a variety of plant-pathogen combinations (for review, see Malamy and Klessig 1992, Uknes *et al.* 1992, Lawton *et al.* 1996). In the majority of cases the term SAR is also used, thus broadening the basic concept in which the inducing agent is a pathogen.

In all cases of the SAR induced by necrotising pathogens or chemical agents described so far, a concomitant accumulation of pathogenesis-related proteins (PR-proteins) was observed. However, the relationship between SAR and PR-proteins may not always be direct (Ye *at al.* 1989, 1992). Originally, five families of PR-proteins, numbered 1 - 5, have been classified (for review, see Linthorst 1991). Within each family, members of the so-called class I are generally localized in the vacuole, whereas class II proteins occur extracellularly. Proteins of the PR-2 family have β-1,3-endoglucanase activity, and those of the PR-3 family have endochitinase activity, but plants expressing either gene alone are not significantly protected. These results suggest that the accumulation of PR-proteins is causally related to the onset of SAR. However, definite evidence for the role of PR-proteins in SAR is still lacking because low contents of these proteins are also present in uninfected plants where their concentrations change during maturation, caryopsis development and germination (Seetharaman *et al.* 1996, Repka *et al.* 1997). Recently, the number of PR-proteins families increased up to fourteen including the proteins found in other plant species (Van Loon and Van Strien 1999).

In an infected host cell, virus RNA can be synthesised from intermediates of the reductive pentosephosphate pathway during photosynthesis or from intermediates of the oxidative pentosephosphate pathway which is active preferentially in the dark, or from intermediates released from degraded host rRNA. These three metabolic pathways are involved in virus RNA biosynthesis, but their participation usually depends on the type of virus, host, and environmental conditions (Šindelářová *et al.* 1997).

The reductive pentosephosphate pathway (the key enzyme is ribulose-1,5-bisphosphate carboxylase/oxygenase) did not differ significantly during the first 10 d post infection between healthy and infected plants, but then a rapidly fall of its rate to 60 - 65 % of healthy control values was found in infected leaves (Šindelářová *et al.* 1997).

The oxidative pentosephosphate pathway metabolises glucose-6-phosphate to ribose-5-phosphate which is necessary for the *de novo* biosynthesis of purine and pyrimidine nucleotides of viral RNA. Glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate: $NADP^+$ oxidoreductase, G6P DH, EC 1.1.1.49) and 6-phosphogluconate dehydrogenase (6-phospho-D-gluconate: $NADP^+$ oxidoreductase, EC 1.1.1.44) play an important role in the regulation of the flux through this pathway at the level of its first enzyme G6P DH (Turner and Turner 1980).

Findings on the influence of virus infection on the rate of the oxidative pentosephosphate pathway have not yet been completely consistent; some authors reported a decreased rate (Bozart 1969), others unchanged rate (Takahashi 1971). However, most research workers explicitly found increased activity of the enzymes involved in the oxidative pentosephosphate pathway (especially of both dehydrogenases), mainly in tissues surrounding local necrotic lesions (*e.g.* Solymosy and Farkas 1962, Huth 1973). The results obtained in our earlier studies also confirmed such increased activity of the whole oxidative pentosephosphate pathway (Šindelář 1986, Šindelář *et al.* 1990, 1999b, Šindelářová *et al.* 1997, 1998).

Knowledge about the number of multiple forms, intracellular location and metabolic functions of the enzymes involved in the degradation of ribonucleic acids in host plant cells is not fully clear (Farkas 1982, Green 1994). The occurrence of an RNA-degrading enzyme activity was demonstrated in extracellular space (Barna *et al.* 1989) but up to 80 % of RNA-splitting capacity was found in intracellular space. Cytochemical and direct isolation studies have revealed the vacuolar location of nuclease activities (Matile 1978). Abel and Glund (1986) and Boller and Kende (1979) indicated that the major vacuolar ribonucleolytic activity was identical with that of the soluble major plant endoribonuclease. The increase in the activity of ribonucleases (RNases) in plant tissues could be induced by wounding (Bagi and Farkas 1967, Diener 1961), by fungal infection (Barna *et al.* 1989, Sutton and Shaw 1982), by chilling or osmotic stress (Kazmierczak and Knypl 1994, Wagih and Coutts 1982) or by viral infections.

Diener (1961) observed stimulation of RNases in TMV-inoculated *Datura stramonium* and in bean pod mottle virus-inoculated *Phaseolus vulgaris*, Wyen *et al.* (1972) found increased concentration of a relatively purine specific endoribonuclease in TMV-inoculated Xanthi-nc tobacco leaves, and Randles (1968) reported that the activity of one of three host RNases rose significantly at the time of rapid virus accumulation in Chinese cabbage systemically-infected with turnip yellow mosaic virus. RNases are not only typical stress enzymes, they represent one of three ways of the virus-RNA biosynthesis from precursors released from degraded host rRNA (Cheo 1971, Reddi 1959, 1963, Šindelář *et al.* 1990, 1999a, Šindelářová *et al.* 1997, 2000a,b). Similarly, the stimulation of phosphomonoesterase (PME), phosphodiesterase (PDE) and other enzymes participating in the degradation of the host rRNA was observed (Šindelářová *et al.* 2000b).

This paper presents the results of the detailed study of changes in control enzymes of host rRNA degradation (RNases, PME, PDE) and of oxidative pentosephosphate pathway (G6P DH) activity in locally and systemically-infected leaf tissue induced by BTH application, and of induced PR-proteins composition.

Materials and methods

Plant cultivation and virus inoculation: Two-month-old tobacco (*Nicotiana tabacum* L. cvs. Samsun and Xanthi-nc) plants grown under constant conditions in Hoagland No. 3 nutrient solution, at an irradiance of 100 μmol m^{-2} s^{-1} (16-h photoperiod) and average temperature of 25 °C, were used. The experimental plants of tobacco were once sprayed with a 1 mM BTH suspension in water. The day of BTH application was designated as day zero post BTH application (0 dpBTH).

Two leaves of the bottom insertion, approximately 5 cm long, were on the 5th day post BTH application mechanically-inoculated with purified tobacco mosaic virus (TMV, common strain) (Gooding and Hebert 1967) at a concentration of 10 μg cm^{-3} (cv. Xanthi-nc) and 100 μg cm^{-3} (cv. Samsun), respectively, and named "locally-infected leaves". Corresponding leaves of control plants were mock inoculated with distilled water. The day of inoculation was designated as day zero post TMV inoculation (0 dpTMV). The upper leaves not directly infected were named as "systemically-infected leaves". The samples of 20 leaves were collected on the designated days and used for analysis.

Preparation of homogenate: Homogenates were prepared from the samples by grinding in a mortar with fine silica sand, 10 % (m/m) insoluble polyvinyl-pyrrolidone and TEMM buffer (20 mM Tris/HCl buffer, 1 mM EDTA, 2.5 mM $MgCl_2$, 0.5 mM PMSF, 1 mM benzamidine, 1 mM ε-aminocaproic acid, 30 mM 2-mercaptoethanol, pH 7.0) in a ratio of 1:5 (m/v). The resulting homogenate was squeezed through Miracloth and nylon sieve 100 mesh and centrifuged for 10 min at 20 000 *g*. Preparation and storage of homogenates were carried out at 0 to 4 °C. Under these conditions the activity of the enzymes did not change for more than 5 h.

Determination of protein and chlorophyll contents and enzyme activities: Soluble protein content was determined according to Bradford (1976) using bovine serum albumin as a standard, and chlorophyll content according to Arnon (1949).

The ribonucleases (RNases) activity assay was a modified procedure of Cheo (1971). Samples containing 0.1 cm^3 of homogenates were incubated with 0.3 cm^3 of yeast RNA (*Serva*, Heidelberg, Germany) (1.33 mg in 1 cm^3 of 0.15 M acetate buffer pH 5.5, resp. 7.0) for 30 - 60 min at 38 °C. At the end of enzymatic reaction the remaining RNA was precipitated with 3 cm^3 of precipitation solution (457.5 cm^3 ethanol, 30 cm^3 glacial acetic acid, 2.25 cm^3 2 M NaOH) for one hour at 0 °C. The mixture was centrifuged for 10 min at 5 000 *g* and the amount of degraded RNA in the supernatant was determined spectrophotometrically at 260 nm. One enzyme unit (U) was defined as the amount of the enzyme causing an increase of 1.0 in the absorbance at 260 nm per hour.

Phosphomonoesterase (PME, EC 3.1.3.2) and phosphodiesterase (PDE, EC 3.1.4.1) activities were assayed using *p*-nitrophenylphosphate or bis-*p*-nitrophenylphosphate as substrates at its pH optima (5.5, resp 6.0) according to Chersi *et al.* (1966). G6P DH (EC 1.1.1.49) activity was determined spectrophotometrically (*Beckman DU-6*, Irvine, USA), NADPH generation was monitored at 340 nm. The assay mixture (0.3 cm^3) contained 0.1 cm^3 of TM buffer (100 mM Tris-HCl buffer, 12.5 mM $MgCl_2$, pH 8.0 which is the pH optimum of the enzyme), 1 μmol of $NADP^+$, 0.1 cm^3 of homogenate and 1 μmol of glucose-6-phosphate. The results obtained for G6P DH activities, have been expressed as the half the generated NADPH in nmol, with respect to a high activity of phosphogluconate dehydrogenase producing an equal amount of NADPH in the assay mixture (Šindelář *et al.* 1999a).

Enzyme activities were determined at their respective pH optima at 25 °C (with the exception 38 °C for ribonucleases).

Determination of TMV content: TMV content was determined by the quantitative DAS-ELISA (Clark and Adams 1977) with rabbit anti-TMV antibodies and alkaline phosphatase labeled antibodies prepared against our isolate of TMV (common strain). Virus content was estimated on the base of calibration curve of purified TMV (Gooding and Hebert 1967) using computer software described by Mančal (1987).

Intercellular fluid extraction and PAGE of proteins: Intercellular fluid (ICF) from freshly harvested leaves was collected by vacuum infiltration using the method as described by Pierpoint *et al.* (1987). Discontinuous nondenaturing polyacrylamide gel electrophoresis (PAGE) in a 1 mm thick 10 % resolving gel and 4 % stacking gel was performed to analyse acidic (Laemmli system) proteins (Hames and Rickwood 1990), using the *Mighty Small II* apparatus (*Hoefer Scientific Instruments*, San Francisco, USA). The amount of proteins loaded to each well corresponded to 6 - 12 μg of proteins. Gel was silver stained (Hames and Rickwood 1990).

Statistical treatment: The results in tables are presented as arithmetical means ± SE of 3 - 7 measurements in four independent experiments. The *t*-test was employed to characterise the differences.

Chemicals: Alkaline phosphatase was obtained from *Boehringer* (Heisenhofen, Germany) and all other biochemicals were purchased from *Sigma Chemical Company* (St. Louis, USA).

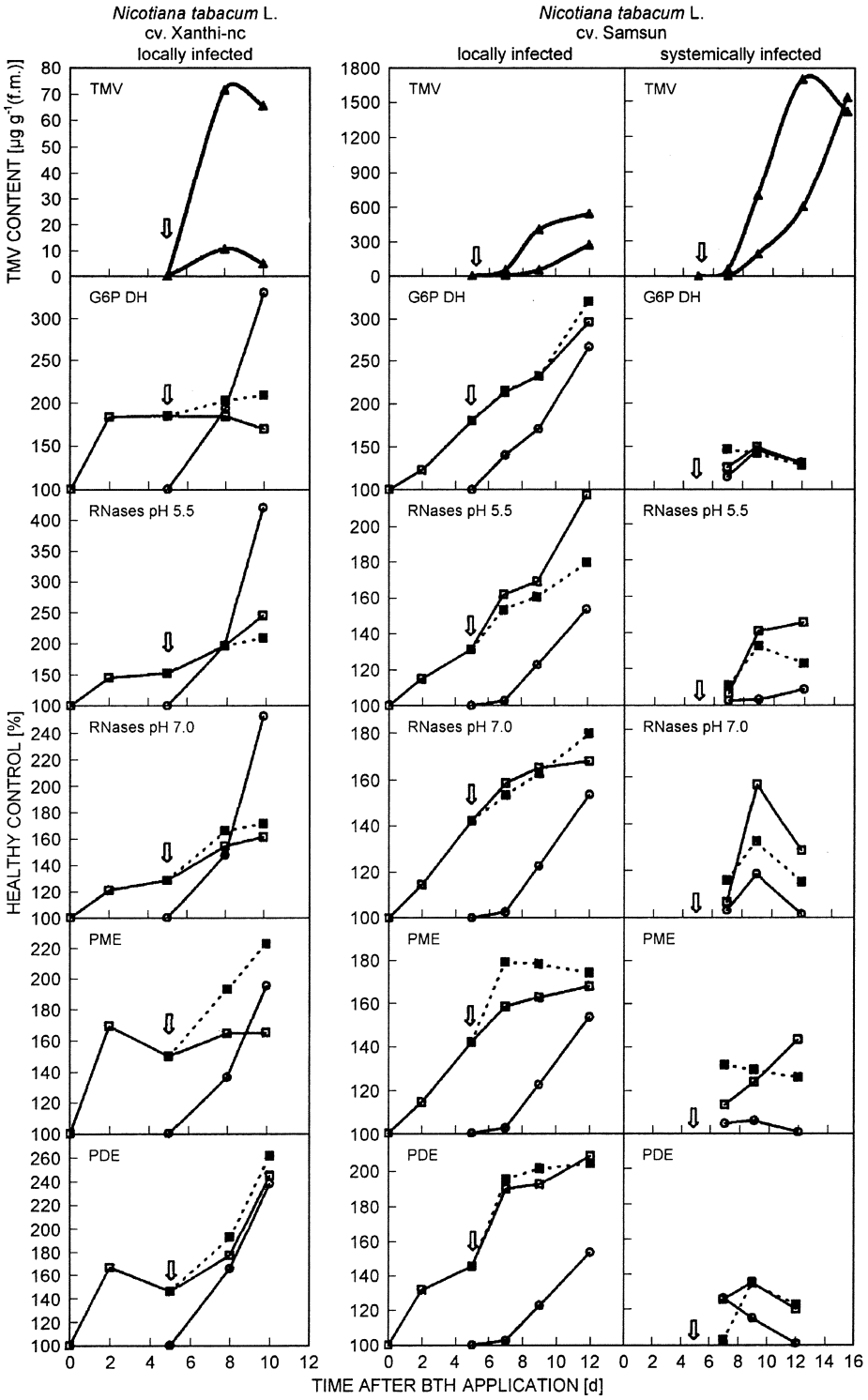

Fig. 1. The TMV content, the glucose-6-phosphate dehydrogenase (G6P DH), the ribonucleases (RNases with pH optima 5.5 and 7.0), the phosphomonoesterase (PME) and the phosphodiesterase (PDE) activities in the locally and the systemically-infected leaves of *Nicotiana tabacum* L. cvs. Xanthi-nc and Samsun. The TMV content is given in µg TMV g^{-1}(f.m.), the enzymes activities are expressed in % of the healthy untreated control. Symbols: The content of the TMV in the BTH pre-treated (*closed triangles*) and untreated (*open triagles*) tobacco cultivars infected with the TMV. The enzymes activities in the TMV-infected (*circles*), the BTH treated (*open squares*) and the BTH pre-treated TMV-infected (*closed squares*) plants. The *arrows* indicate the day of the TMV inoculation (on the 5^{th} day after BTH application).

Table 1. Chlorophyll and soluble protein contents [mg g^{-1}(f.m.)], G6P DH [nmol g^{-1}(f.m.) s^{-1}], RNases (pH 5.5 and 7.0) [U g^{-1}(f.m.)], PME and PDE [μmol g^{-1}(f.m.) s^{-1}] activities in healthy mock inoculated and locally infected leaves of *Nicotiana tabacum* L. cv. Xanthi-nc after BTH treatment (BTH). TMV inoculated (TMV) and TMV inoculated on the 5th day after BTH treatment (BTH + TMV).

	Healthy					BTH				TMV			BTH + TMV			
dpBTH	0	2	5	8	10	2	5	8	10	5	8	10	5	8	10	
dpTMV			0	3	5		0	3	5	0	3	5	0	3	5	
chlorophyll	0.613	0.613	0.622	0.668	0.696	0.748	0.628	0.663	0.701	0.622	0.673	0.709	0.628	0.672	0.691	
proteins	3.890	3.980	3.950	3.990	3.840	4.700	4.760	4.850	4.620	3.950	4.030	3.910	4.760	3.920	3.820	
G6P DH	0.388	0.390	0.416	0.422	0.315	0.716	0.770	0.781	0.537	0.416	0.819	1.042	0.770	0.858	0.660	
RNase pH 5.5	27.200	27.540	31.450	66.470	53.380	39.740	47.800	130.900	131.100	31.450	131.700	224.600	47.800	130.700	111.800	
RNase pH 7.0	25.840	29.750	35.840	52.870	47.940	36.030	46.120	81.840	77.520	35.840	78.250	121.100	46.120	87.920	82.360	
PME	72.100	87.200	120.700	147.100	131.500	147.600	181.100	242.400	217.400	120.700	201.100	257.100	181.100	284.500	293.300	
PDE	9.820	10.410	13.930	13.740	9.710	17.350	20.380	24.390	23.810	13.930	22.820	23.170	20.380	26.550	25.400	

dpBTH - day post BTH application

dpTMV - day post TMV inoculation

Table 2. Chlorophyll and soluble protein contents [mg g^{-1}(f.m.)], G6P DH [nmol g^{-1}(f.m.) s^{-1}], RNases (pH 5.5 and 7.0) [U g^{-1}(f.m.)], PME and PDE [μmol g^{-1}(f.m.) s^{-1}] activities in healthy mock inoculated and locally/systemically infected leaves of *Nicotiana tabacum* L. cv. Samsun after BTH treatment (BTH), TMV inoculated (TMV) and TMV inoculated on the 5th day after BTH treatment (BTH+TMV).

Local infection

	Healthy						BTH					TMV				BTH+TMV			
dpBTH	0	2	5	7	9	12	2	5	7	9	12	5	7	9	12	5	7	9	12
dpTMV			0	2	4	7		0	2	4	7	0	2	4	7	0	2	4	7
chlorophyll	0.69	0.70	0.67	0.59	0.58	0.58	0.70	0.79	0.59	0.59	0.57	0.67	0.59	0.59	0.57	0.79	0.78	0.58	0.57
proteins	3.37	3.72	3.63	3.58	3.74	3.58	3.78	4.32	4.38	4.43	4.29	3.63	3.56	3.75	3.60	4.32	4.39	4.41	3.62
G6P DH	0.39	0,41	0,42	0.36	0.58	0.16	0.50	0.76	0.78	0.64	0.47	0.42	0.51	0.47	0.42	0.76	0.78	0.64	0.51
RNase pH 5.5	28.90	34.00	43.35	71.06	53.55	46.58	39.03	56.83	114.90	90.45	101.40	43.35	72.84	65.65	71.55	56.83	108.90	85.95	83.75
RNase pH 7.0	20.40	23.12	30.09	42.84	32.13	33.15	26.47	42.76	67.86	53.05	55.66	30.09	43.91	39.38	50.92	42.76	65.63	52.24	59.60
PME	117.80	119.70	122.70	111.60	125.30	93.60	137.10	174.40	176.80	203.90	157.20	122.70	114.40	153.60	143.80	174.40	210.30	223.30	163.10
PDE	12.32	13.23	15.20	14.55	12.82	6.71	17.44	22.12	27.67	24.70	13.99	15.20	14.91	15.72	10.31	22.12	28.45	25.85	13.71

Systemic infection

	Healthy				BTH			TMV			BTH+TMV		
dpBTH		7	9	12	7	9	12	7	9	12	7	9	12
dpTMV		2	4	7	2	4	7	2	4	7	2	4	7
chlorophyll		0.80	0.86	0.88	0.81	0.85	0.879	0.81	0.86	0.88	0.81	0.86	0.87
proteins		4.46	4.87	4.71	4.41	4.89	4.750	4.45	4.83	4.71	4.40	4.89	4.77
G6P DH		0.61	0.52	0.60	0.77	0.77	0.790	0.70	0.76	0.79	0.89	0.74	0.77
RNase pH 5.5		72.76	76.75	70.41	77.27	108.10	102.400	74.58	79.05	76.46	80.69	101.80	86.43
RNase pH 7.0		37.57	38.90	46.24	40.05	61.03	59.650	38.70	46.17	46.98	43.62	51.62	53.36
PME		189.70	200.50	172.50	215.10	248.80	248.100	198.80	212.90	174.20	166.20	260.10	218.00
PDE		19.20	18.29	17.02	24.12	24.78	20.460	24.29	21.03	17.19	19.74	24.67	20.90

dpBTH - day post BTH application
dpTMV - day post TMV inoculation

Results and discussion

The multiplication of the TMV was studied either in the hypersensitively-reacting cultivar Xanthi-nc, which generates the necrotic lesions at the site of infection, or in the sensitive cultivar Samsun where the infection is spread systemically from the inoculated leaves (local infection) to upper non-inoculated leaves (systemic infection). The multiplication curve of the TMV culminated in the locally-infected leaves of the cultivar Xanthi-nc on the 3rd day post inoculation and the maximum of TMV content was 71.7 μg TMV g^{-1}(f.m.). Pre-treatment with BTH caused that the content of virus was strongly reduced to 10.8 μg TMV g^{-1}(f.m.); it is of about 15 %, respectively. In the locally-infected leaves of the tobacco cv. Samsun, the TMV content steadily increased to the maximum of 547 μg TMV g^{-1}(f.m.) on the 7th day post inoculation (corresponding to the 12th day of application of the BTH, 12 dpBTH). The BTH application reduced the content of virus to 275 μg TMV g^{-1}(f.m.) equal to 50.3 %. The extensive multiplication of TMV was also observed in the systemically-infected leaves, with the maximum of 1690 μg TMV g^{-1}(f.m.) on the 10 dpTMV. However, the decrease in the multiplication of TMV caused by the BTH application was observed only for initial period of infection (Fig. 1). At the end of experiment, the TMV content of BTH pre-treated tobacco was similar to the value, which was found in systemically-infected leaves of non-treated tobacco. The observations suggest that multiplication of the TMV is only delayed not suppressed in this pre-treatment.

Chlorophyll content was not significantly changed by infection with TMV either in the cv. Samsun or cv. Xanthi-nc. After application of BTH, a small increase (by about 20 %) in the chlorophyll content was observed both in the leaves of cv. Xanthi-nc and cv. Samsun. Similarly, the BTH treatment initiated a mild enhancement in soluble protein content (Tables 1, 2).

The TMV inoculation induced an increase in the activity of the glucose-6-phosphate dehydrogenase (G6P DH) up to 330 % in the tobacco cv. Xanthi-nc on the 10 dpTMV. The BTH treatment likewise increased the activity of this enzyme up to 180 % soon after the application. However, the subsequent inoculation with virus on the 5 dpBTH resulted only in an additional increase in the activity (of about 17 % on the 12 dpTMV). Analogous results were observed in the locally-infected leaves of cultivar Samsun with exception that progressive infection evoked the continuous increase in the activity even in the BTH pre-treated tobacco (Tables 1, 2, Fig. 1). In systemically-infected leaves of cv. Samsun, a much lower increase in the activities was found.

Activities of RNases (pH optimum 5.5 and 7.0) were increased in either the locally- or systemically-infected leaves of both cultivars. The BTH treatment alone enhanced the activities of these enzymes, as well. But in contrast to the G6P DH, the TMV infection following the BTH pre-treatment did not change or even decreased the activities of RNases with pH optimum of 5.5 when compared to BTH treatment alone (Tables 1, 2, Fig. 1).

The trends of the PME and PDE activities appear similar to that of RNases however; in particular the PME seems to be more increased subsequently to the inoculation even in the BTH pre-treatment (Tables 1, 2, Fig. 1).

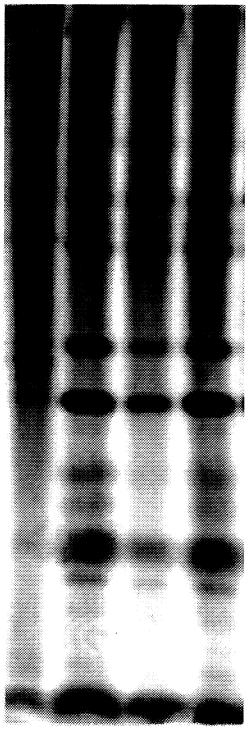

Fig. 2. Electrophoretic analysis of acidic extracellular proteins from locally-infected leaves *Nicotiana tabacum* L. cv. Xanthi-nc (silver-stained) in 10 % polyacrylamide gel under native conditions (Laemmli system) extracted on the 7th day post inoculation: *lane 1* - mock-inoculated untreated leaves, *lane 2* - TMV-inoculated leaves, *lane 3* - BTH-treated leaves, *lane 4* - BTH-treated and TMV-infected leaves.

Inoculation of tobacco with TMV evoked the enhancement of all studied enzymes in either susceptible or hypersensitive cultivar. This is in agreement with observations of Diener (1961), Huth (1973), Randles (1968), Takahashi (1971) and with our previous experiments (Šindelář 1986, Šindelářová *et al.* 1997, 1998, 2000a,b, 2001). The BTH treatment likewise considerably increases these activities. In the BTH pre-treated tobacco, the subsequent infection with TMV induced only a weak additional increase of about 14 - 17 % in the activities of some enzymes (especially

G6P DH, RNases 7.0 and PDE in locally-infected leaves of both cultivars), decrease of about 10 - 30 % in others (RNases pH 5.5 and 7.0, PME in systemically-infected leaves of cultivar Samsun and RNases 5.5 in locally-infected leaves of both cultivars) or no changes were found (G6P DH and PDE in systemically-infected leaves of cultivar Samsun). On the base of the results we suppose a competition between effect of BTH and TMV infection in pools of substrates of metabolic pathways leading to biosynthesis of viral RNA (Tables 1, 2, Fig. 1).

The direct effect of the BTH on enzymes activities involved in the TMV-RNA biosynthesis was studied *in vitro* by measuring the activities with addition of the BTH (concentrations from 1 to 1000 μM) into spectrophotometric cuvette. No effect on the enzymes activities was found with the exception of 1 mM BTH (concentration used for spraying of experimental plants) that inhibited only the G6P DH to 36 % (Table 3). Therefore, the increase in studied enzymes induced by BTH *in vivo* is not likely caused by the direct action of BTH.

The separations of the intercellular proteins from cultivar Xanthi-nc by PAGE under native conditions show similar spectrum of proteins extracted both from the BTH-treated tobacco and the TMV-infected tobacco (Fig. 2). In cultivar Samsun, only the BTH treatment (not the TMV infection) induced the synthesis of PR-proteins (data not shown).

Table 3. Influence of the BTH on the *in vitro* activity of control enzymes of metabolic pathways for TMV-RNA biosynthesis. The values in parentheses express the percentage of the healthy BTH nontreatment control.

Enzyme activity	BTH concentration [μM] 1000	100	10	1	0
G6P DH	0.214	0.577	0.590	0.596	0.602
[nmol g^{-1}(f.m.) s^{-1}]	(35.5)	(95.8)	(98.0)	(99.0)	(100.0)
PME	164.3	184.6	197.8	200.0	185.2
[μmol g^{-1}(f.m.) s^{-1}]	(88.7)	(99.7)	(106.8)	(108.1)	(100.0)
PDE	19.16	19.73	19.70	20.23	19.16
[μmol g^{-1}(f.m.) s^{-1}]	(100.0)	(103.0)	(102.8)	(103.2)	(100.0)
RNases pH 5.5	68.17	75.65	70.01	72.62	70.5
[U g^{-1}(f.m.)]	(96.7)	(107.3)	(99.4)	(103.0)	(100.0)
RNases pH 7.0	33.61	36.00	35.31	34.91	36.14
[U g^{-1}(f.m.)]	(93.0)	(99.6)	(97.7)	(96.6)	(100.0)

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