Susceptibility of tobacco leaves of different age to the infection with the potato virus X (PVX) and its nucleic acid (PVX-RNA)

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INTRODUCTION

The reliability of the local lesion test as a method for measuring the concentration of active virus in a given preparation largely depends on the uniformity of plant material used as test plants. One of the most important features of this uniformity is the age of inoculated leaves, especially in those cases when the whole leaf inoculation design is applied.

The different susceptibility of leaves of different age to virus infection by means of mechanical inoculation is generally recognised [1]. In many virus — host combinations older leaves are less susceptible than the young ones (eg. PVX on *Chenopodium amaranticolor* or *Ch. quinoa*), in others — the opposite appears to be true (e. g. PVX on *Gomphrena globosa*), unless the inoculated leaves are already too old and begin to decay.

As far as the reaction of tobacco plant (*Nicotiana tabacum*) is concerned, higher susceptibility of older leaves to the infection with PVX was stated in the variety Samsun after inoculation with the ringspot strain of this virus [13]. The author observed a similar relationship when testing different PVX preparations on *N. tabacum* variety White Burley. As successive leaves of that variety differ largely in their size, the obtained lesion counts must be recalculated versus the leaf area in order to obtain comparable numbers.

EXPERIMENTAL

Table 1 summarises results of testing 24 different PVX samples (the ringspot strain) on three successive leaves of tobacco seedlings *N. tabacum* variety White Burley. In the majority of cases the inocula represented crude buffer extracts obtained from PVX infected tobacco leaves. Purified preparations were made from the same material by chloroform extraction procedure according to Corbett [3]. It may be seen, that apart of only two samples (No. 10 and 13), which gave unusually high numbers of lesions per leaf, being therefore not reliable for comparison of infecti-

Number of local lesions on leaves of different age of tobacco (calculated per 100 sq.

Sample	Date of experiment	Inoculum: source plants: virus symptoms	Inoculum: source plants: days after inoc.	Inoculum: dilution and/or extraction media	Inoculum: concentration (dilution)
1	2	3	4	5	6
A. Crude sap (*N. tabacum variety* White Burley)					
1	7.V	L	14	phosphate buffer	1:100
2	„ „	„	„	0.025M: pH 7	„ „
3	„ „	„	„	„ „	„ „
4	„ „	„	„	„ „	„ „
5	3.XI	L	3	1% pyrophosphate	„ „
6	„ „	„	„	„ „	„ „
7	6.XI	L	4	„ „	„ „
8	„ „	„	„	„ „	„ „
9**	7.XI	„	5	„ „	„ „
10	„ „	„	„	„	„ „
11	„ „	„	„	„ „	1:1000
12	„ „	„	„	„ „	„ „
13	23.X	S	22	„ „	1:100
14	„ „	„	„	„ „	„ „
15	„ „	„	„	„ „	„ „
16	20.X	S	24	1% K_2HPO_4	„ „
17	22.II	S	21	phosphate buffer	„ „
18	„ „	„	„	0.025M, pH 7	1:1000
B. Purified preparations**					
19	15.II			phosphate buffer 0.025M, pH 7	0.0027%
20	„ „			„ „	„
21	„ „			„ „	„
22	„ „			„ „	„
23	19.II			„ „	0.0005%
24	„ „			„ „	„

* Expressed as percentage of the corresponding value of the top leaf.
** Lesions counted in UV light.

vity, all the remaining samples produced as a rule significantly less lesions per unit of the leaf area on top leaves of the inoculated tobacco plants, than on the two lower situated leaves. The lower susceptibility of the top leaf (youngest leaf) to the infection with PVX appeared to be independent on such characters of the inoculated virus preparations as the degree of their purification, age of infection of the source plants and the kind of buffer solution used for the preparation of inocula, as well

Table 1

variety. White Burley inoculated with the potato virus X, cm leaf area)

post extraction treatment***	Top leaf		Bottom leaf		Middle leaf	
	lesions per 100 sq. cm	leaf area sq. cm	lesions %*	leaf area %*	lesions %*	leaf area % *
7	8	9	10	11	12	13
I	59	130.0	161	56	190	36
II	76	126.0	162	53	110	40
III	48	130.0	190	60	360	36
IV	30	130.0	170	54	233	37
I	134	27.1	97	119	128	123
IV	124	30.2	107	115	100	102
I	201	28.2	285	136	190	124
IV	264	28.8	165	115	195	109
I	253	26.8	138	112	74	86
IV	440	28.0	70	107	53	118
„	224	28.2	114	127	—	—
VI	215	34.5	124	86	—	—
I	1,200	36.1	85	98	58	111
IV	403	27.6	233	159	238	194
VI	538	34.0	125	126	—	—
I	263	24.5	130	140	151	159
I	163	67.9	226	148	206	198
I	118	72.0	119	130	126	160
II	177	80.0	106	61	162	41
III	161	80.0	137	51	248	42
IV	155	84.0	147	53	207	44
V	216	79.0	99	68	119	37
I	32	84.0	182	99	222	47
II	48	81.0	171	91	162	43

*** I — kept at 4° C, II — kept at room temperature, III — kept 1/2 h at 36° C, IV — kept 1 h at 36° C, V — kept 2 h at 36° C, VI — incubated with 50 μg/l RNase for 20 min at room temp. Each number represents an average from 4-6 leaves.

as their different post-extraction treatments (Table 1, columns 3-7). It also seemed to be independent on the size of the inoculated leaves, and of the period of the year in which the experiment was performed (Table 1, columns: 2, 9, 11, 14). Thus, it should be probably regarded as an intrinsic property of the infected leaf itself, depending mainly, if not solely, on its age.

An opposite tendency was stated by inoculations with preparations of PVX—RNA

Number of local lesions on leaves of different age of tobacco variety. White Burley inoculated

Sample	Date of experiment	Type of phenol extract		Top leaf		Middle leaf	
		origin	preparation	lesions	leaf area sq. cm	lesions %*	leaf area %*
1	20.X	S	crude — 1 extr. cycle	97	26	112	124
2	„ „	„	„ — 2 extr. cycle	224	23	76	132
3	22.X	„	„ — 1 extr. cycle	211	22	84	142
4	23.X	„	„ „ „	285	25	33	179
5***	„ „	„	„ „ „	238	31	52	101
6	7.XI	„	„ „ „	166	35	57	87
7	6.XI	L	„ „ „	139	31	136	100
8	5.XI	„	„ „ „	160	31	94	110
9	„ „	„	purified-ethanol	195	28	61	97
10****	30.IV	„	crude-1 extr. cycle	8	42	16	186

* Expressed in percentage of the corresponding value of the top leaf.
** Expressed as percentage infectivity of the corresponding crude sap preparation.
*** Extracted from crude sap incubated 1 h at 36° C.

(Table 2). In the majority of cases they represented crude phenol extracts, obtained from PVX infected tobacco leaves according to the procedure described by Schlegel [16]. Only one sample contained RNA previously purified by ethanol precipitation. 7 out of 10 tested samples produced significantly more lesions per unit of the leaf area on top leaves of the inoculated plants, than on the middle or bottom leaves. As the PVX—RNA lesions were usually chlorotic and diffuse, they were counted under UV light. It may be seen from the data, that the higher susceptibility of younger leaves to infection with PVX—RNA was also apparently independent on the provenience of inocula, their degree of purification, and the period of the year within which the experiment was carried out.

This unexpected behaviour of PVX—RNA preparations was further studied by comparing their infectivities with infectivities of the corresponding PVX samples out of which the RNA inocula were obtained. As can be seen on Fig. 1 the relative infectivity values of different PVX—RNA samples were usually much lower when calculated at middle or bottom leaves of the inoculated tobacco plants as compared with the top leaves. The opposite took place only in those cases in which also the susceptibility of the lower leaves to the infection by PVX—RNA was apparently higher (Table 2).

The different reaction of tobacco leaves of various age to the infection with PVX could be also observed when the inocula were previously incubated at 36° C for 0.5-1hr. In earlier experiments (unpublished data) it was shown, that this treatment often considerably increased the infectivity of the tested PVX preparations, but sometimes its effect was just opposite. In order to get idea what might be the reason of such discrepancies dilution infectivity curves, obtained after and before

Table 2

with undiluted phenol extracts of PVX-RNA, (calculated per 100 sq. cm leaf area)

Bottom leaf		Relative infectivity**				Relative infectivity in % of the top leaf value	
lesions %*	leaf area %*	top leaf	middle leaf	bottom leaf	average	midle leaf	bottom leaf
183	140	0.37	0.32	0.44	0.38	87	119
69	145	0.82	0.50	0.39	0.57	61	48
58	182	—	—	—	—	—	—
53	173	0.71	0.11	0.16	0.33	15.5	22.5
59	117	0.59	0.13	0.15	0.29	22	25
154	93	0.66	0.27	1.36	0.61	41	205
—	—	0.69	0.33	—	0.41	48	—
—	—	1.20	1.16	—	1.09	97	—
—	—	1.45	0.91	—	1.18	63	—
—	—	4.80	1.60	—	3.60	34	—

**** Extracted in phosphate buffer pH 7, 0.025 m; L — inoculated leaves with local lesion, S — leaves with systemic symptoms.

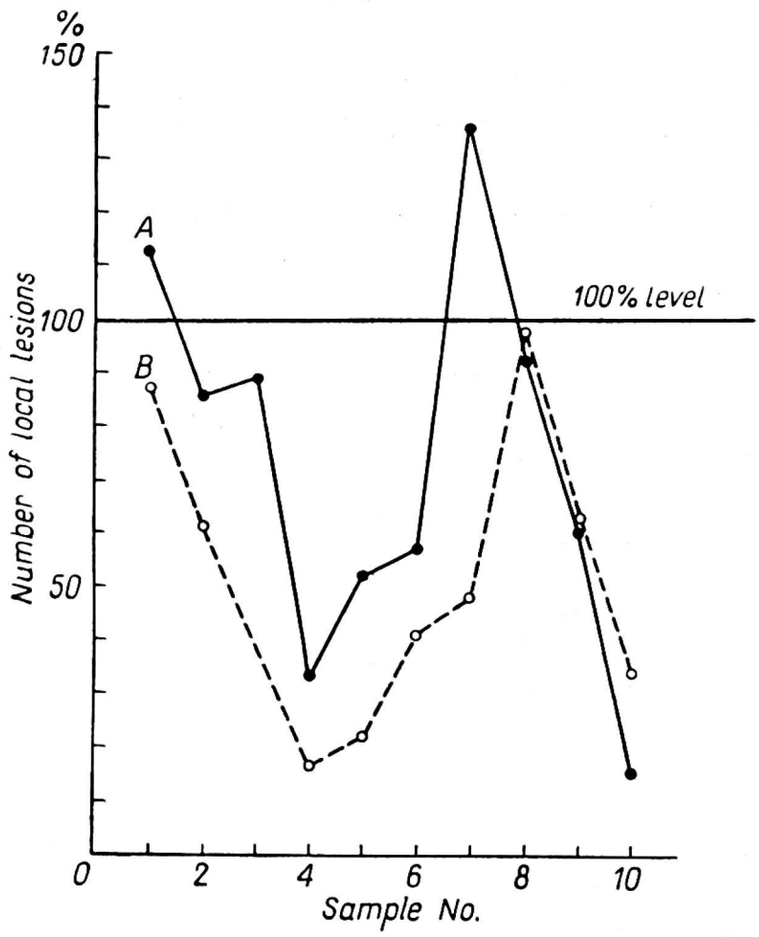

Fig. 1. The relation between changes in number of local lesions (curve *A*) and relative infectivity values (curve *B*) obtained on the middle leaves of *N. tabacum* variety White Burley after inoculation with different samples of PVX—RNA. Expressed in per cent of the corresponding values for the top leaf.

heat treatment of a purified PVX preparation, were studied (Figs. 2-5). When we put the data on a logarithmic scale (log. number of lesions versus log. virus concentration) usually a characteristic curve is obtained which at high virus concentrations approaches asymptotically the theoretical maximal possible number of local lesions, which can be formed under constant conditions [4, 12]. As can be seen on Fig. 2

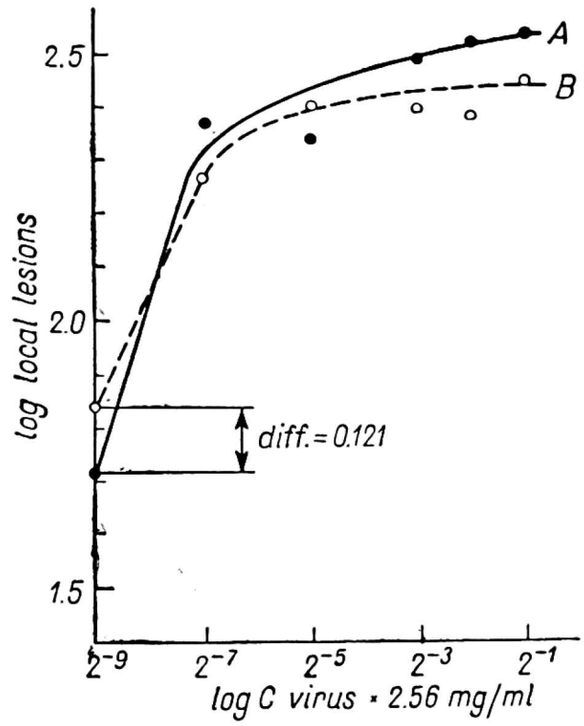

Fig. 2. Effect of incubation of purified PVX preparation at 36°C for 1/2 hr on its dilution infectivity curve. *A* — control sample, *B* — sample incubated at 36°C.

this maximum number of lesions was much lower for PVX preparations incubated for half an hour at 36°C (curve *B*), than for control sample (curve *A*); (280 and 340 lesions per 100 sq. cm leaf area respectively) Both curves: *A* and *B* represent here an average calculated from all inoculated leaves. When only leaves of the same position at the plant (approximately of the same age) are considered, we may see, that although the same general relationship between both curves was maintained, the difference between maximal possible numbers of lesions, produced by the heat treated and control inocula, increased steadily from top to the bottom leaves (Fig. 3-5). On the contrary, at high dilutions heat treated inocula were averagely more infectious than the control samples, this difference being most obvious at the top leaves, but rather insignificant at bottom leaves of the inoculated tobacco plants.

This characteristic dependence of infectivities of both kinds of PVX inocula on their dilution, appeares still more clearly if appropriate values of percentage inactivation of heat treated PVX preparation are computed for different dilutions (Table 3). They are calculated according to Utech and Johnson [18] from the following formula $\frac{A-B}{A} \times 100$; where *A* — denotes the number of local lesions produced by the control sample, and *B* — the same produced by the treated sample. It may be seen, that at high dilutions the percentages attain negative values what means, that the

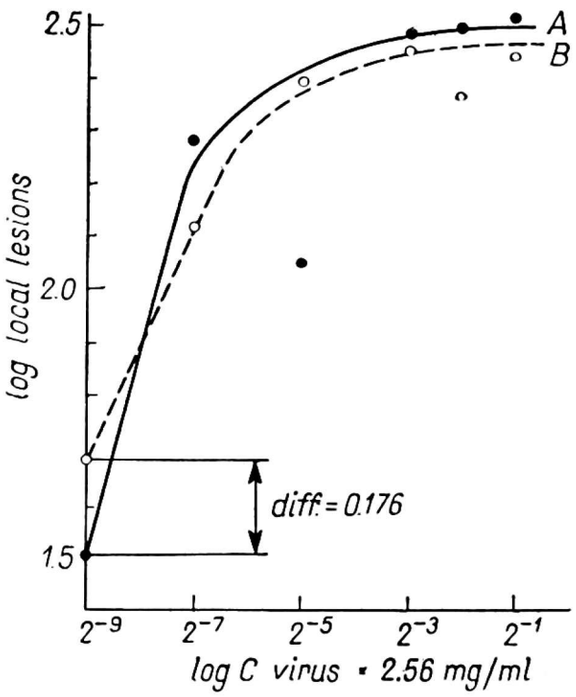

Fig. 3. Effect of incubation of purified PVX preparation at 36°C for 1/2 hr, on its dilution infectivity curve on top leaves of *N. tabacum* variety White Burley. *A* — control sample, *B* — sample incubated at 36°C.

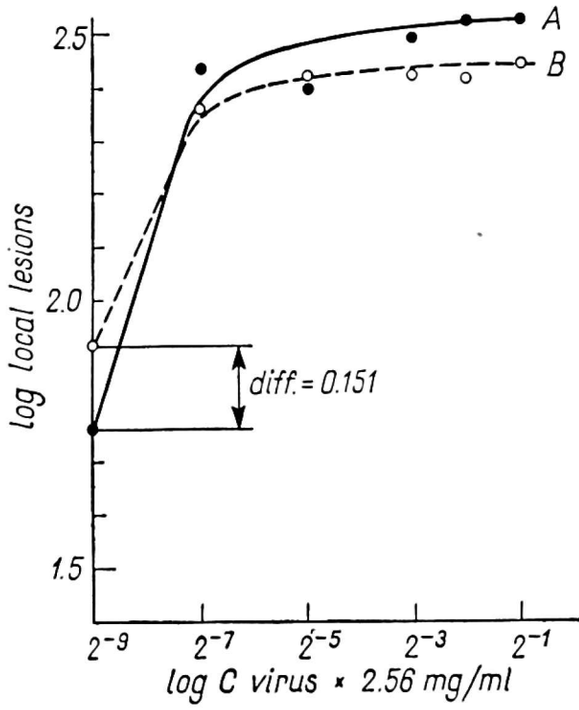

Fig. 4. Effect of incubation of purified PVX preparation at 36°C for 1/2 hr, on its dilution infectivity curve on middle leaves of *N. tabacum* variety White Burley. *A* — control sample, *B* — sample incubated at 36°C.

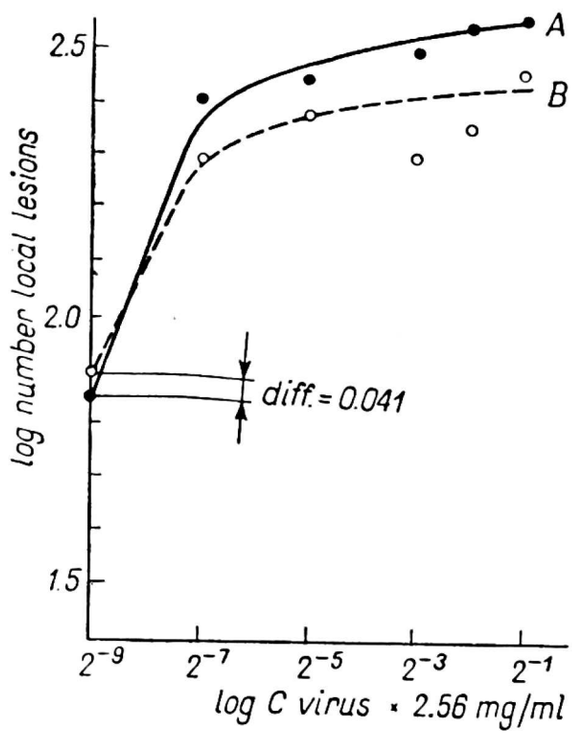

Fig. 5. Effect of incubation of purified PVX preparation at 36°C for 1/2 hr, on its dilution infectivity curve on bottom leaves of *N. tabacum* variety White Burley. *A* — control sample, *B* — sample incubated at 36°C.

infectious potency of the heat treated sample was here greater, than that of the control sample. This difference reveales however a tendency to disappear at the bottom leaves (smallest percentage of activation).

Table 3

Percentage inactivation of heat treated PVX preparation as estimated on the top, middle and bottom leaves of *N. tabacum* variety White Burley at different dilutions. (Each number represents an average from 4-6 leaves)

Dilution	Leaf position			
	top	middle	bottom	average from 3 leaves
2^{-1}	15.4	17.4	21	17
2^{-2}	25	21	34	27
2^{-3}	5	16.5	37	19
2^{-5}	—118	—3.5	15	—15
2^{-7}	30	14.5	19	20
2^{-9}	—50	—41	—10	—32

The limiting dilutions above which the activation effect of the heat treatment can be detected are in each case determined, as can be seen from the graphs, by the points of interesection of each pair of infectivity curves, and these are shifted much towards higher dilutions for older leaves. This explains why in certain cases, when the assays were made on such leaves, the stimulating effect of heating on infectivity of PVX preparations was not observed (unpublished data). The incubated samples produced here often less lesions, than the control, probably because the virus concentration in the compared inocula was to high.

DISCUSSION

Following mechanisms could be considerred as possible causes of the different behaviour of the examined inocula when tested on tobacco leaves of different age: Higher susceptibility of older leaves to PVX infection could result from the general shift in metabolism of senescent tissues with retarded or completely restrained growth, what under certain conditions may promote virus multiplication [5, 8, 20]. In fact, as was shown by Reddi [14], older tobacco leaves exhibit higher ribonuclease activity, than the young ones, what might lead to increased hydrolysis of host RNA and accumulation of building blocks (nucleotides) for the subsequent synthesis of viral particles [6]. On the other hand the same hydrolytic activity of ribonuclease could account for the lower infectivity of PVX—RNA preparations, observed on older leaves [2, 11]. Similar mechanisms may at least partly explain the effects of high temperature on the PVX infectivity dilution curves. It is very probable, that heating makes virus aggregates, which are normally present in inocula, more readily dispersable upon dilution in comparison with unheated samples. Hence the increased infectivity of such inocula at high dilutions [7, 17]. On the other hand, as was shown on the graphs (Figs. 2-5), the same inocula revealed much lower maximum infectivity level, than the control ones, kept at 4°C. We may suppose, that the heat treated samples contained more inert protein particles, resulting maybe from partial inactivation of virus material on heating, which could act as inhibitors

of infection [9, 15]. This effect of inhibition would then be much more pronounced at high virus concentrations [1, 10], and still increased in older leaves which usually contain more inhibitors of viral infection themselves [19]. It is also possible, that heating of PVX preparations at 36°C produces a high percentage of highly labile virus particles, sensitive to inactivation by leaf ribonuclease. Inactivation of such particles would proceed at higher rate by inoculation of older tobacco leaves, which exhibit an increased activity of this enzyme [11, 14].

Taking into consideration all those effects we may conclude, that at high virus concentrations the largest difference in lesion counts between heat treated and control inocula was found at the oldest inoculated leaves, probably because the infectivity of the treated sample was decreased both by the presence of inert protein (inhibition effect) and the action of ribonuclease on labile PVX particles (inactivation effect). On the contrary, at high dilutions (low virus concentration), where is usually no effect of inhibition of infectivity, caused by extra viral constituents of the inocula [1], the difference in infectivity between both kinds of compared PVX preparations appeared to be smallest when tested on oldest tobacco leaves. It may be argued, that in this case the effect of increased PVX infectivity, resulting from the dispersion of virus aggregates on heating was suppressed by the increased hydrolytic activity of ribonuclease in older leaves, which inactivated the majority of dissagregated virus particles.

SUMMARY

Three consecutive leaves of tobacco seedlings *N. tabacum* variety White Burley were inoculated by different PVX preparations and with RNA extracts obtained from tobacco plants infected with the same virus. It was found, that the bottom leaves of infected tobacco plants were more susceptible to PVX infection, than the top leaves, but were less susceptible to the infection with RNA preparations. PVX preparations incubated at 36°C for 0.5-1hr showed an increase in infectivity in comparison with the control samples when tested at high dilutions, this difference being most pronounced on the top leaves. On the contrary, when tested at high virus concentrations, the heat treatment resulted in a decrease of infectivity of PVX preparations, this decrease being most obvious at bottom leaves of inoculated tobacco plants. The author supposes, that those effects resulted from combined activity of such factors determining the infectivity of a given virus preparation as: the dispersion of virus aggregates in preparations on heating, the increase of amount of highly labile, ribonuclease sensitive PVX particles as well as of inert extra viral material in heat treated samples, and finally the increased hydrolytic activity of ribonuclease in older leaves.

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