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Effect of Kinetin on Lesion Development and Infection Sites in Xanthi-nc Tobacco Infected by TMV: Single-cell Local Lesions

By

E. BALÁZS, B. BARNA and Z. KIRÁLY

Department of Pathophysiology, Research Institute for Plant Protection, H-1525, Budapest P. O. Box 102, Hungary

Both the crude and the ultracentrifuged extracts of the kinetin-treated Xanthi-nc tobacco leaf tissues were more infective than was expected on the basis of the number of local lesions caused by TMV. In fact, infectivity of 100 lesions from the kinetin-treated half leaves exceeded that of 100 lesions from the control halves. The infective RNA content of the kinetin-treated half leaves did not change in spite of the significant reduction in lesion number. The virus did not become systemic as a result of kinetin treatment. By selective staining of necrotic cells in tissues with Evans blue it was shown that the actual number of local lesions was not reduced as a result of kinetin treatment. This treatment suppresses only the number of necroses visible to the naked eyes, but more necrotic single cells were seen on the kinetin-treated half leaves under the microscope.

It was shown by several investigators that cytokinins, such as kinetin or benzyladenine, may influence virus infection as well as virus multiplication (SELMAN, 1964; KIRÁLY and POZSÁR, 1964; KIRÁLY and SZIRMAI, 1964; DAFT, 1965; ALDWINCKLE and SELMAN, 1967; MILO and SRIVASTAVA, 1969; KIRÁLY *et al.*, 1968; ALDWINCKLE, 1975).

The results from several laboratories seem to be rather contradictory concerning the effect of cytokinins on lesion number in local lesion hosts and on virus infectivity. Some authors have reported on the reduction of virus local lesions (both number and size) and also on the reduction of infectivity per unit leaf area or fresh weight. Conversely, reports were also published showing that lesion number and infectivity could be stimulated by the administration of cytokinins to leaves.

Because we were the first who reported on the suppression of the virus in a local lesion host by kinetin (KIRÁLY and SZIRMAI, 1964), we wanted to have a deeper insight into the mechanism of reduction of lesion number and infectivity of tobacco mosaic virus (TMV) in Xanthi-nc tobacco. Our aim was to compare the reduction in lesion number in kinetin-treated half leaves with the reduction of virus infectivity of crude leaf extracts of the kinetin-treated halves as well as with the ultracentrifuged leaf extracts. Also, in untreated (control) half leaves the infectivity of TMV in the developed local lesions was compared to that of the kinetin-treated halves and the infective TMV-RNA was determined in the kinetin

treated half leaves in comparison with the control values. It seemed also indispensable to know more about the local lesion development on a cellular level in the kinetin-treated tissues and a possible systematization of the virus in the kinetin-treated leaves.

Materials and Methods

Virus and plants

Nicotiana tabacum L. cv. Xanthi-nc was grown under ordinary greenhouse conditions and used for experiments in the 10-leaf-stage. The Ul strain of TMV was cultured in *Nicotiana tabacum* L. cv. Samsun. Tobacco leaves infected with TMV were ground (1 g leaf per 10 ml tap water) with a pestle and mortar and the homogenate used to inoculate the leaves of experimental tobacco plants. No abrasive was added to the inoculum. At various intervals after kinetin treatment the whole leaf was inoculated with TMV by use of a glass rod.

Kinetin treatment

Kinetin (Calbiochem) at 20 μg/ml tap water was applied twice daily for 10–20 days by spraying the attached half leaves of Xanthi-nc tobacco plants. In each experiment we used 12 plants, and on each plant 6–8 leaves.

Virus infectivity

Virus infectivity in leaves of treated and untreated half leaves was deter mined by the local lesion assay. The infectivity of virus preparation was deter mined by inoculating leaves of *N. tabacum* cv. Xanthi-nc and counting the loca lesions produced. Samples, 5 g each, were taken from leaves which had been inoculated with TMV 48 h previously. Before extractions were made, the surface of the leaves was thoroughly washed with 2% NaOH and rinsed with running tap water for 5 min to eliminate possible surface contaminations. The samples for assay were ground with precooled mortar and pestle containing 12 ml 0.06 *M* phosphate buffer pH 7, and 0.05% 2-mercaptoethanol. Leaf homogenates were centrifuged at 5000 *g* for 30 min at 0°C and the supernatant was assayed on Xanthi-nc tobacco. These supernatants were further centrifuged at 105 000 *g* for 60 min at 4°C. The pellet was resuspended in 0.06 *M* phosphate buffer pH 7,0 or in the supernatants, and then assayed on Xanthi-nc.

Infective RNA

Infective RNA was extracted by the phenol method (FRAENKEL-CONRAT *et al.*, 1961). Five-five g samples were homogenized in 2 ml GPS buffer (0.1 *M* glycine, 0.05 *M* K_2HPO_4, 0.3 *M* NaCl, adjusted to pH 9.5) containing 1 ml of

10% bentonite and 5 ml water-saturated phenol. The homogenate was stirred for 45 min at 4°C. After centrifugation at 6000 *g* for 45 min at 0°C the water phase was extracted and an equal volume of phenol containing 1 ml of 10% bentonite was added. The RNA was precipitated by addition of two volumes of cold ethanol. After 2 h at −20°C the suspension was centrifuged at 6000 *g* for 30 min at 0°C. The supernatant was discarded and the precipitate was dissolved in 1 ml GPS buffer containing 0.5 ml 10% bentonite. To assay for infectivity two drops of the samples were dropped onto Xanthi-nc leaves and rubbed with a glass rod.

Staining with Evans blue

Experimental tobacco leaves were infiltrated with 1% aqueous solution of Evans blue (Fisher Scientific Company, St. Louis) at various intervals after virus inoculation. This pigment was infiltrated into the inoculated leaves with a hypodermic syringe, and, after a 15 min equilibration period, discs (12 mm in diameter) were examined by light microscopy (TURNER and NOVACKY, 1974).

Results

Infectivity of crude leaf extracts and the ultracentrifuged extracts in comparison with the reduced local lesion number

Half leaves of Xanthi-nc tobacco were sprayed with 20 ppm kinetin solution twice daily for 10–20 days before inoculation with TMV. This procedure proved to be successful in suppressing the number of local lesions on the kinetin-treated half leaves as compared to the water-sprayed control halves. In a series of experiments the extent of the reduction of local lesions was different and always dependent on the length of period of the application of kinetin. In each experiment infectivity of the kinetin-treated and water-treated half leaf extracts and that of the ultracentrifuged extracts was also determined. Table 1 shows the extent of reduction in lesion number as a result of kinetin treatment and the extent of reduction or stimulation in infectivity of the same kinetin-treated half leaf as well as the changes in infectivity after ultracentrifuging the extract of the same leaf material. As is seen, the suppression of local lesion number corresponds with changes in infectivity of the crude extracts and that of the ultracentrifuged material. However, the crude extract and particularly the ultracentrifuged extract of the kinetin-treated tissues were always more infective than was expected on the basis of the number of local lesions. It was also found that the infectivity of the ultracentrifuged extracts of the kinetin-treated tissues was about the same or higher than that of the water control when the reduction in lesion number was low.

We can not explain the increased infectivity of the ultracentrifuged extract of the kinetin-treated half leaves as compared to that of the crude extract. We

Table 1

Kinetin-induced changes in lesion number and in virus infectivity of TMV in Xanthi-nc tobacco

Experiments	Period of kinetin treatment in days	Lesion number in per cent of untreated half leaves*	Infectivity of crude extracts in per cent of untreated half leaves*	Infectivity of ultracentrifuged virus in per cent of untreated half leaves*
I.	18—20	28.9**	53.0	79.0
II.	14—17	44.2	70.8	86.8
III.	10—13	60.3	94.0	127.3

Each experiment consisted of 5 replications. In each replicate we used 12 plants and on each plant 6—8 leaves. The samples were assayed on 20 leaves of Xanthi-nc by the half leaf method.

* The correlation between lesion number and infectivity of the crude extract was highly significant ($P < 0.01$); between lesion number and infectivity of the ultracentrifuged extract the correlation was significant ($P < 0.05$).

** Mean number of lesions per half leaf for the water-sprayed control was 175.

tested for inhibitors in the supernatants of the ultracentrifuged extracts but did not find any additional inhibitory effect on virus infection when they were compared to water as control. More research is needed to find an explanation for this phenomenon.

It would seem, that the kinetin treatment actually increases the infectivity of virus in lesions which were able to develop. It seems logical to suppose that, as a result of kinetin treatment, more infective particles exist in local lesions or perhaps in the tissues between local lesions.

Table 2

Infectivity of TMV in lesions and in area between lesions after kinetin treatment

Experiments	Lesion number			
	Untreated		Kinetin-treated*	
	100 lesions	Area between lesions	100 lesions	Area between lesions
I.	111	0	145	0
II.	98	0	131	0
III.	163	0	199	0
IV.	165	0	248	0

Hundred-hundred samples were cut from the leaves with cork-borer (2 mm in diameter), ground in 3 ml buffer pH 6.9 and assayed on Xanthi-nc.

* Plants were treated with 20 ppm kinetin solution for 14 days.

Table 3

TMV-RNA infectivity in control and in kinetin-treated Xanthi-nc tobacco leaves

Experiments	Infectivity of TMV-RNA Lesion number	
	Untreated half leaves	Treated half leaves
I.*	45	47
II.*	63	67
III.*	52	52

In each experiment we treated 12 plants on each 6—8 leaves. The samples were assayed on 8—10 leaves of Xanthi-nc.

* The ratio of lesion numbers on control and kinetic-treated half leaves were 100 : 40 (I. experiment); 100 : 45 (II. experiment) and 100 : 46 (III. experiment), respectively.

It was experimentally determined that the infectivity of virus particles of 100 lesions from the kinetin-treated half leaves indeed exceeded infectivity of 100 lesions from the control halves (Table 2). Additionally, in evaluation of samples taken from tissues between local lesions, it was convincingly demonstrated that the virus did not become systemic as a result of the action of kinetin on suppressing the number of the visible lesions.

Table 3 shows the infective RNA content of the kinetin-treated half leaves in comparison with the water-treated control halves. In spite of the significant reduction in lesion number in the kinetin-treated half leaves, the infective RNA content did not change. One can suppose that the higher virus-RNA content of lesions of the kinetin-treated halves compensates for the reduction in lesion number.

Influence of kinetin treatment on the number of infection sites and on the suppression of necrotization

It is seen from the foregoing that more infective particles and more viral-RNA exist in local lesions as consequences of kinetin treatment, thus they compensate for the suppression of infection sites. This seems to be the situation on a macroscopic level, however, Figs 1 and 2 show that this is not the case on a microscopic level. We infiltrated 1.0% aqueous solution of Evans blue into the intercellular space of the leaves after inoculation at various time intervals, and searched for dead cells using a microscope. Evans blue selectively stains the necrotic cells in tissues (GAFF and OKONG'O-OGOLA, 1971). Surprisingly, many more necrotic single cells were seen on the kinetin-treated and virus-inoculated half leaves, than on the untreated but inoculated ones. In other words, the number of infection sites and the number of local lesions were not reduced by kinetin

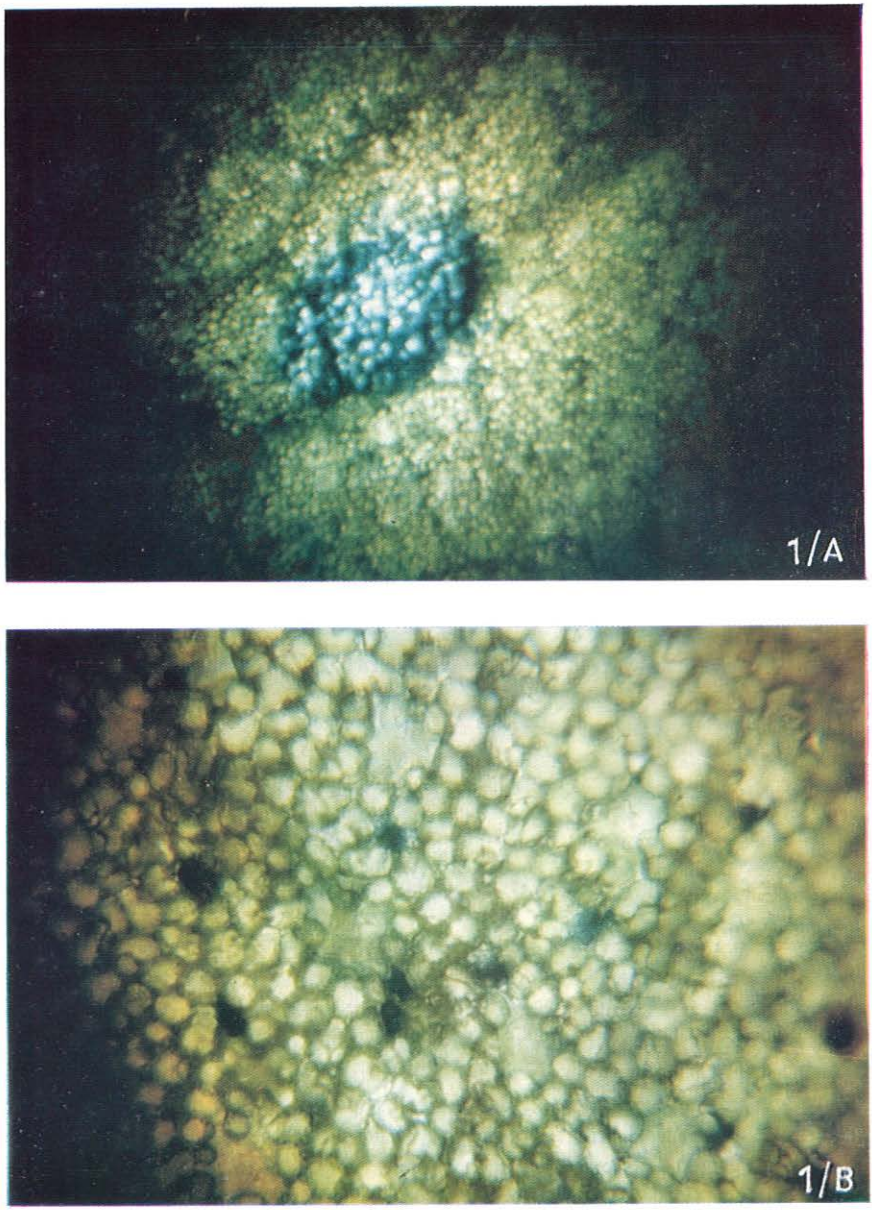

Fig. 1. Necroses induced by TMV in Xanthi-nc tobacco leaves (stained by Evans blue 48 h after inoculation). A: Visible local lesion on the control half leaf. B: Single-cell necroses on the kinetin-treated half leaf

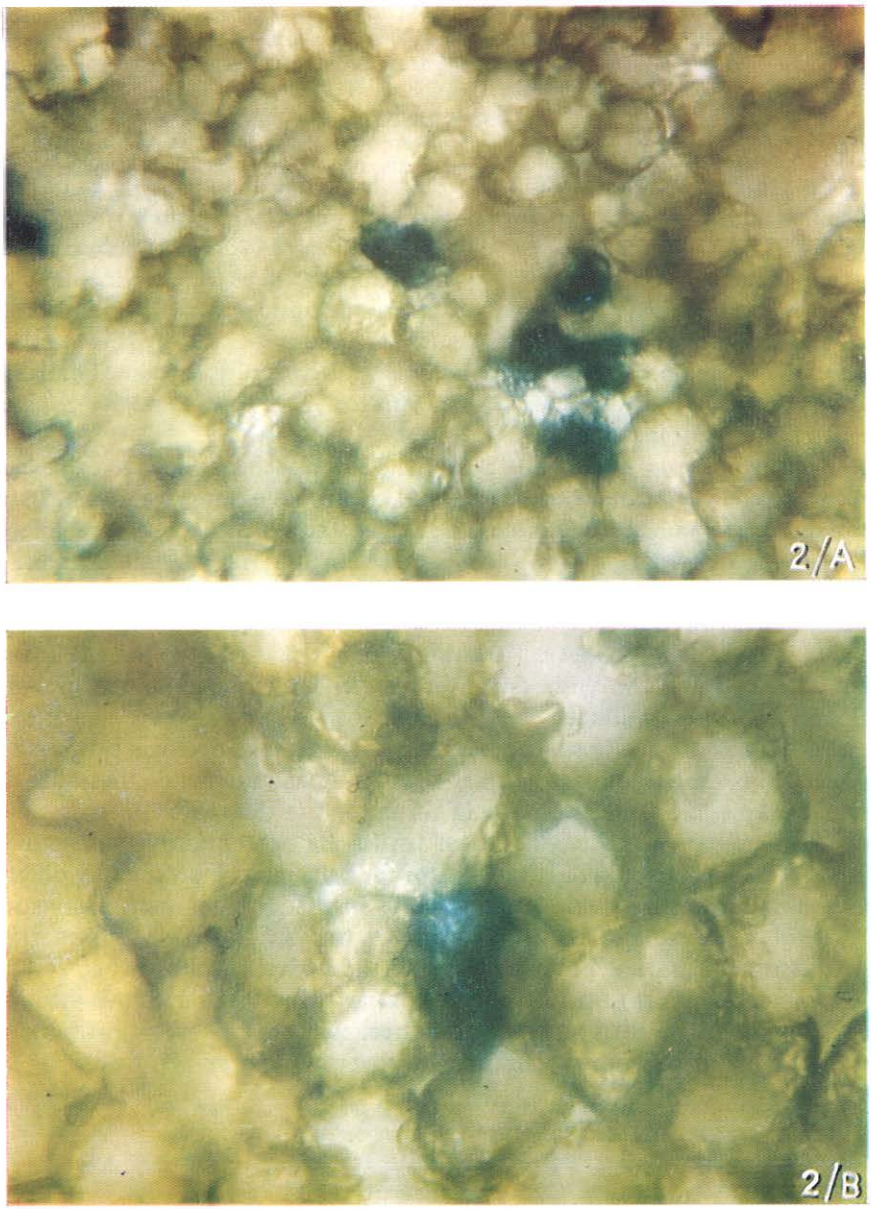

Fig. 2. Single-cell necroses on the kinetin-treated half leaf at different magnifications. A: A group of one-cell local lesions. B: A single-cell local lesion

treatment, only the size of the local lesions was influenced. The kinetin treatment suppresses only the number of necroses visible to the naked eyes, but the actual local lesion number is the same on the kinetin-treated and on the control half leaves.

Discussion

In a recent paper, ALDWINCLE (1975) called attention to the relation of timing of the first application of cytokinins to the inhibitory or stimulatory effect on virus infectivity. According to his theory, the application of cytokinins before inoculation may render host metabolism less susceptible to infection, e.g. virus take-over will slowing down under circumstances where the normal host RNA and protein syntheses are stimulated by cytokinins (cf. KIRÁLY *et al.*, 1968). However, after successful inoculation, the cell machinary is mostly used to produce viral RNA and viral protein, and then the cytokinin treatments might stimulate virus synthesis (cf. ALDWINCKLE and SELMAN, 1967).

This hypothesis, although in a modified form, seems to be applicable in our case where the treatment with kinetin took place only before virus inoculation. By reducing the number of visible lesions, kinetin seems to exert an inhibitory effect on TMV multiplication (in tissue area between visible lesions we were not able to show virus multiplication by bioassay). On the other hand, we found stimulation of virus infectivity in lesions of the kinetin-treated half leaves. Applying the speculation of ALDWINCKLE (1975) one can hypothesize that some cells in the tissue are very active in virus take-over and in this instance kinetin treatment will stimulate virus synthesis. This tissue area more or less corresponds to the areas of visible necrosis. In some other cells, the virus take-over will slowing down or even inhibited because the normal host RNA and protein syntheses are stimulated by kinetin. These areas would correspond to the one-cell necroses in the leaf. One can suppose that cells in leaf tissues are not uniformly susceptible to infection because the physiological state of the cells may be profoundly different. The non-uniform physiological state of cells in tissues have been demonstrated by several workers (cf. ATKINSON and MATTHEWS, 1967; MATTHEWS, 1970). Indeed ultrastructural and physiological differences of cells relate to a fine "mosaic" in the tissue (cf. WOOLHOUSE, 1967). It is clear to us that the above-mentioned speculation is a hypothesis with very few direct evidences. However, with the help of this hypothesis one can explain the controversial data on this field.

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