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The rôle of cytokinins in the systemic acquired resistance of tobacco hypersensitive to tobacco mosaic virus

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Cytokinin content increased in Xanthi tobacco leaves systemically resistant to challenge inoculation by tobacco mosaic virus (TMV). Subsequently, root formation was strongly suppressed and shoot formation was promoted in leaf cultures derived from resistant leaves. All of these events coincided with the time of development of systemic acquired resistance, i.e. 8 days after the primary inoculation of the lower leaves. In spite of a reduction in the number of visible local lesions in resistant upper leaves, infectivity of ultracentrifuged TMV did not change markedly. Following a 1 day treatment at high temperature (32 °C), when necrotization is inhibited and TMV becomes systemic, this type of resistance was not manifested. It is suggested that only the necrotization is suppressed in challenge-inoculated resistant leaves, and the production of the virus is not reduced. Chloramphenicol and ethrel, which promote local lesion production, were counteracted in leaves in which systemic acquired resistance had been induced.

INTRODUCTION

The mechanism of systemic acquired resistance [*16*, *17*], the failure of lesions to develop to normal size in upper non-inoculated tobacco leaves following lesion formation in lower leaves of TMV-inoculated hypersensitive tobacco, still remains to be elucidated. The nature of the factor(s) systemically distributed from the primarily infected leaves, as well as the mechanism of inhibition of tissue necroses (lesions) and virus multiplication in the systemically resistant leaves, are unknown. The putative rôle of polyphenoloxidase and of peroxidase [*16*, *20*] was criticized and rejected [*5*, 26]. Van Loon [*25*] tried to correlate the development of acquired resistance in the upper leaves with the appearance of several specific proteins after inoculation of the lower leaves of hypersensitive tobacco. However, it was pointed out by this author that systemic acquired resistance was also expressed in the upper parts of tobacco plants in which tissue necroses were induced in the lower leaves by spraying with mercuric chloride instead of TMV-inoculation, in this case no new protein components were induced. Thus the explanation of the rôle of proteins, determined by disc electrophoresis, seems to be unsatisfactory.

Our recent work [*4*], based on earlier investigations, demonstrated that exogenously applied kinetin suppressed the development of local lesions induced by inoculation of Xanthi tobacco with TMV; while the number of infective virus particles was not changed, visible lesions were smaller and numerous non-visible single-cell lesions (necroses) were produced. In addition, working with leaf cultures

made from healthy, TMV-infected and from Xanthi tobacco leaves having the systemic acquired resistance phenomenon, we often experienced in preliminary tests a striking suppression of root formation. The inhibition or suppression of root formation was detected only in TMV-infected leaf cultures and in those that derived from non-infected but systemically resistant upper leaves. As is known, organ formation in tissue culture depends on cytokinin : auxin ratios in the substrates and in plant tissues.

These preliminary findings drew our attention to the possible rôle of cytokinins in the mechanism of systemic acquired resistance of tobacco to TMV. The objectives of the present study were to determine the changes of endogenous cytokinins during the development of systemic acquired resistance, to discover more about the possible rôle of cytokinins in suppression of visible local lesions after challenge inoculation, and to determine the infective virus content in leaves in which the systemic acquired resistance developed. We also tried to suppress resistance in the upper leaves by the application of chemicals which are antagonistic to cytokinins.

MATERIALS AND METHODS

Plant material and virus inoculum

Tobacco plants (*Nicotiana tabacum* cv. Xanthi-nc) were grown under normal glasshouse conditions and were used for experiments at the 8 to 10 leaf stage. The U_1 strain of tobacco mosaic virus (TMV) was cultured in *Nicotiana tabacum* cv. Samsun. Tobacco leaves showing the typical disease symptoms of TMV were ground (1 g leaf per 10 ml 0·1 M phosphate buffer) with a pestle and mortar and the homogenate used to inoculate the test plants. No abrasive was added to the inoculum. The primary infection was made on the lower three or four leaves, and the remaining upper leaves were used for challenge infection.

Treatments

Ethrel (2-chloroethylphosphonic acid, Amchem Prod. Inc., Ambler, Pa, U.S.A.), 200 μg/ml in 0·1 M phosphate buffer, pH 6·5, was sprayed on one-half of each uninoculated Xanthi leaf 7 days after the first inoculation of the lower leaves. The treatments were made twice daily and the challenge inoculation was made 10 days after the first infection. Chloramphenicol (Sigma, St Louis, U.S.A.), 200 μg/ml, was similarly applied in a parallel experiment.

Cytokinin extraction and bioassay

At 3, 8 and 42 days after the first inoculation, samples (30 g fresh weight) from healthy and from inoculated tobacco plants were taken and macerated in a Waring Blendor with 50 ml 0·15 M phosphate buffer, pH 6·5, and then extracted with 300 ml of methanol for 12 h at 4 °C. The cytokinin extraction was made as described earlier [*23*]. After the extraction, the residue was dissolved in 96% ethanol, streaked onto Whatman No. 1. chromatography paper, and separated with different solvent systems (*n*-butanol : concentrated NH_4OH : water, 3 : 1 : 1 v/v/v and ethylacetate : formic acid : water, 60 : 5 : 35, upper phase). After drying, the chromatograms were divided into ten equal strips and tested for cytokinin activity in the soybean

callus bioassay. The method of Miller [*14*] modified by Krasnuk *et al.* [*11*] was used. Cultures were maintained at 27 °C for 28 days and then weighed.

Virus infectivity

After challenge inoculation, virus infectivity in control and systemically resistant leaves was determined by the local lesion assay on Xanthi-nc tobacco. Samples, 10 g each, were taken from leaves which had been challenge 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 (cf. [*4*]). The samples for assay were ground with a pre-cooled mortar and pestle containing 20 ml 0·06 M phosphate buffer, pH 7·0, and 0·05% 2-mercaptoethanol. Leaf homogenates were centrifuged at 5000 g for 30 min at 0 °C and the supernatants were further centrifuged at 105 000 g for 60 min at 4 °C. The pellet was resuspended in phosphate buffer (0·06 M, pH 7·0) and then assayed on Xanthi-nc.

All experiments were repeated 8 to 10 times. Samples were taken in each experiment from eight control plants and from eight plants in which systemic resistance developed.

Leaf culture test

Three halves of lower leaves were inoculated with TMV, and at 3, 8 and 42 days after infection the opposite halves and the upper uninoculated leaves were harvested and washed with tap water, rinsed in 2% NaOCl solution containing 0·1% Tween 20 for 20 min, and then washed with sterile water three times. After this sterilizing procedure, small squares (approximately 5 × 5 mm) were cut from the leaves and put into Erlenmeyer flasks which contained basal medium [*13*], containing 0·2 mg/l indoleacetic acid (IAA) and different kinetin concentrations (0·0, 0·03, 0·6, 1·2, 2·4 mg/l). Each flask contained four leaf squares. After 28 days shoot and root formation was assessed.

Effect of high temperature on manifestation of systemic acquired resistance

Seven or 10 days after the primary inoculation the upper leaves were challenge inoculated and afterwards plants were placed at 32 °C for 24 h with continuous light (3600 lux). After 48 h the lesion number was counted and compared to that of the control plants.

RESULTS

Alterations in organ formation of cultures from leaves having the systemic acquired resistance

Leaf squares (5 × 5 mm) cut from control and resistant leaves 8 days after inoculation were put on a basal medium containing 0·2 mg/l IAA and a series of different concentrations of kinetin. It is seen in Plate 1 that root formation was strongly suppressed in resistant leaf cultures whether they derived from the opposite half-leaves of TMV-inoculated half-leaves (a) or from the upper leaves (b). Initiation of shoot formation in the case of control leaf cultures (c) occurred only at very high concentrations of kinetin (1·2, 2·4 mg/l). On the other hand, shoot formation was promoted

even at 0·6 mg/l kinetin concentration when samples were taken from resistant leaves. At this relatively low concentration of kinetin, cultures from the control leaves produced only roots.

The above-mentioned alterations in organ formation did not occur 3 and 42 days after inoculation, only 8 days after the initial TMV inoculation. It is known from the experiments of Ross [*16*] and Bozarth & Ross [*6*] that the induction of systemic acquired resistance begins 3 to 4 days after inoculation, reaching a maximum in about 7 days, and then disappears 42 days after inoculation. Thus, the suppression of root formation as well as the initiation of shoot formation at low kinetin concentration in leaf cultures coincides with the development of resistance in leaf tissues. This coincidence suggested an investigation of the putative rôle of cytokinin in the induction of systemic acquired resistance.

Cytokinin content of resistant leaf tissues

Cytokinins of control and resistant upper leaves were extracted and separated by paper chromatography and bioassayed in the soybean callus tissue culture test. Samples were taken 3, 8 and 42 days after inoculation of the lower leaves. There was no considerable difference in the cytokinin content of control and resistant leaves, when samples were taken 3 and 42 days after inoculation. However, 8 days after inoculation of the lower leaves, when the systemic acquired resistance reaches the highest level in the upper leaves, the cytokinin content of the latter significantly increased (Fig. 1). It is seen in this figure that the increase in cytokinins can be demonstrated at R_F values 0·1, 0·3 and 0·7 to 0·8. The materials at R_F 0·1 and 0·3 yielded activities almost in the region to which free zeatin or zeatin riboside migrates. The material at 0·7 to 0·8 would correspond to 2-isopentenyladenine (2iP) and 2-isopentenyladenosine (IPA). The lack of complete coincidence of activities with those of standards does not detract from the point.

Local lesion number and virus infectivity in resistant leaves

In spite of the significant reduction in the visible local lesion number of systemically resistant upper leaves 8 days after the primary inoculation of the lower leaves, the infectivity of the ultracentrifuged TMV does not change markedly (Table 1). The situation is similar to that experienced earlier [*4*] when kinetin was applied exogenously to Xanthi leaves. Both in leaves that were treated with kinetin exogenously and in which the cytokinin level increased endogenously the virus infectivity was not altered, although the development of local lesions was inhibited. In both cases the exact cause remains to be seen. However, it is tempting to suppose that only the extent of necrotization is suppressed and neither the production of TMV nor the number of infection sites in the resistant leaves is reduced. It is interesting to note in this context that in preliminary microscopic tests we always experienced an increase in the number of the non-visible single-cell local lesions. This was also the case with leaves exogenously treated with kinetin [*4*].

That the action of systemically acquired resistance in the upper leaves is on the development of necrotization and not on the virus *per se* was shown in another experiment (Table 2). It is seen in Tables 1 and 2 that virus production in resistant leaves

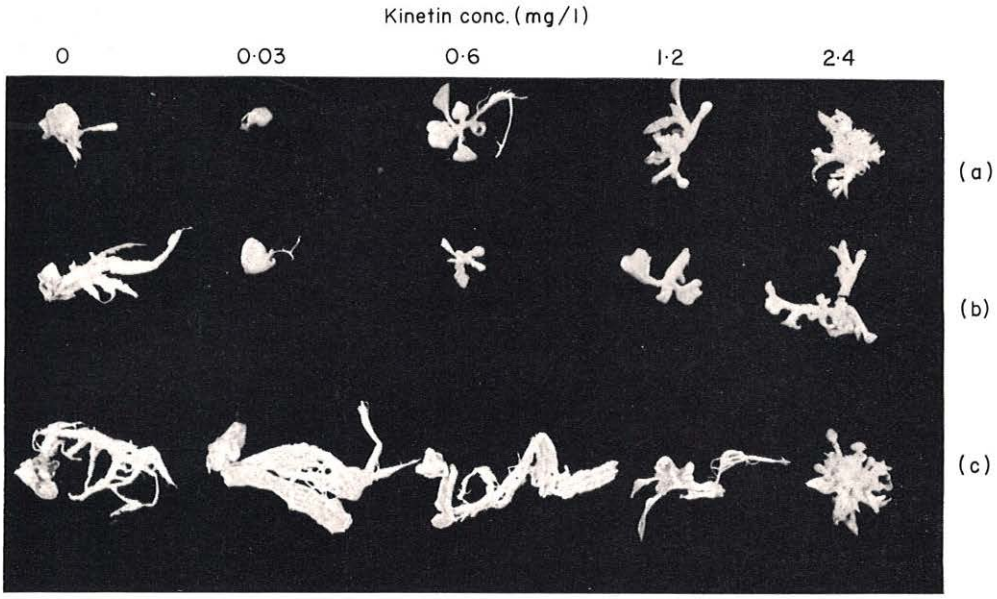

PLATE 1. Organ formation (at different concentrations of kinetin in the medium) by tobacco leaf cultures derived from leaves having the systemic acquired resistance to TMV. (a) Non-inoculated half-leaf opposite to the TMV-inoculated half-leaf, 8 days after inoculation; (b) non-inoculated upper leaf 8 days after inoculation of the lower leaves; (c) leaf derived from healthy tobacco plant.

[*facing page 32*]

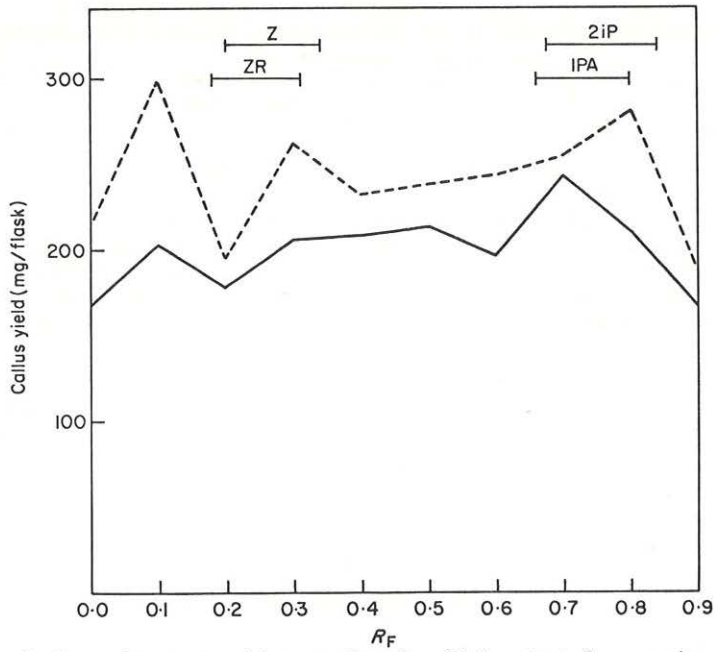

FIG. 1. Paper chromatographic separation of cytokinin extracts from non-inoculated upper Xanthi leaf 8 days after inoculation of the lower leaves with TMV, and from leaf derived from healthy tobacco plants. Samples were chromatographed on Whatman No. 1. paper in ethyl-acetate : formic acid : water, 60 : 5 : 35 upper phase. Cytokinin standards were co-chromatographed. Zeatin (Z); zeatin-riboside (ZR); 2-isopentenyladenine (2iP); 2-isopentenyladenosine (IPA). Soybean callus cultures were grown on basal medium supplemented with portions of chromatograms corresponding to R_F regions. Data represent the average of five replications. (——) Control; (- - - -) resistant.

TABLE 1

Changes in lesion number and in virus infectivity of TMV induced by challenge inoculation in Xanthi-nc tobacco 8 days after the first inoculation

Experiment number	Lesion number in resistant leaves (% control)	Infectivity of ultracentrifuged TMV (% control)
I	53·2	91·2
II	41·5	84·9
III	51·1	90·9
IV	47·4	89·3

Mean number of lesions per leaf for the control plant was 197. The correlation between lesion number and infectivity of ultracentrifuged extract was significant ($P = 0{\cdot}05$).

Each experiment consisted of three replications, and in each replicate we used eight plants and on each plant six leaves. The samples were assayed on ten leaves of Xanthi-nc by the half-leaf method.

was not altered, only the number of visible lesions was reduced. However, the number of local lesions remained unaltered when resistant leaves were challenge inoculated, placed at 32 °C for 24 h (conditions under which necrotization did not develop and the virus became systemic) and afterwards transferred to 24 °C. Under

TABLE 2
The effect of heat treatment on lesion number induced by challenge inoculation in Xanthi-nc leaves having systemic acquired resistance

Plant material	Lesion number/leaf	
	At 24 °C	At 32 °C[a]
Control plant	76	52
Resistant plant	36	49

Data represent the average lesion number on leaves of five replications. In each replication five leaves were taken from each of the four experimental plants (20 leaves).
[a] Seven or 10 days after the primary inoculation the upper leaves were challenge inoculated and then plants were placed in 32 °C for 24 h. Lesion number was counted 48 h after the challenge inoculation.

low temperature, where necrotization could proceed normally, the local lesion development (necrotization) was significantly reduced.

The effect of chloramphenicol and ethrel on the visible local lesions

Both chloramphenicol and ethrel were previously shown to increase local lesion number [*3, 10, 18*]. When we sprayed both compounds on halves of upper leaves during the development of acquired resistance, the visible lesion number was always increased. The extent of the increase was about 70% both in the control and in the resistant leaves. Both compounds were seen earlier to be able to counteract the action of exogenously applied cytokinin on local lesions caused by TMV. Now we have shown that systemic acquired resistance is able to counteract the action of both ethrel and chloramphenicol supposedly by a cytokinin effect (Table 3).

TABLE 3
The effect of chloramphenicol and ethrel on lesion number induced by challenge inoculation in Xanthi-nc leaves having the systemic acquired resistance

Treatment	Lesion number on Xanthi-nc half-leaves after challenge inoculation	
	No ethrel	*Ethrel*[a]
Control	98	168
Resistant (10 days after the first infection)	51	84
	No chloramphenicol	*Chloramphenicol*[b]
Control	98	164
Resistant (10 days after the first infection)	50	81

Data represent the average lesion number on half-leaves of five replications. In each replication five leaves were taken from each of the four experimental plants (20 leaves).
[a] Ethrel (200 μg/ml) in 0·1 M phosphate buffer, pH 6·5, was sprayed on one-half of each uninoculated leaf 7 days after the first inoculation of the lower leaves. Treatments were made twice daily and the challenge inoculation was made 10 days after the first inoculation.
[b] Chloramphenicol (200 μg/ml) was similarly applied as under ethrel treatment.

DISCUSSION

It has been known for a long time that some exogenously applied cytokinins suppress viral local lesions in tobacco and in other plants [*1*, *2*, *4*, *8–10*, *15*, *19*]. Recently it was shown by Sziráki & Balázs [*21*] that the cytokinin level increases in a local lesion host of TMV. As regards systemic virus infections, results seem to be contradictory [*12*, *22*]. In the present study it was demonstrated that 8 days after inoculation of the lower leaves of Xanthi tobacco with TMV, the cytokinin content of the non-inoculated upper leaves significantly increased. One can suppose that cytokinins are systemically distributed out of the primarily infected lower leaves toward the upper non-inoculated leaves that thereby acquire resistance to the challenge inoculation. However, it is not possible to tell at present whether cytokinins are indeed translocated from the lower infected leaves to the non-inoculated ones, or are produced in the non-inoculated leaves as a consequence of some unknown stimulus.

Although the increased cytokinin level and the development of acquired resistance in the upper resistant leaves are only correlative phenomena, we suggest that a cause-and-effect relationship exists between them. This seems highly probable, because increase in cytokinins coincides with a high degree of resistance 8 days after the first inoculation. On the other hand, 3 days after the first inoculation, when time was short for the development of resistance, and 42 days after the primary inoculation, when systemic acquired resistance of upper leaves had already disappeared [*6*], the level of cytokinins remained unaltered in the non-inoculated upper leaves. It is not possible at present to tell with certainty whether or not appearance of specific proteins in the upper leaves [*25*] is correlated with an increased cytokinin level. Cytokinins may increase proteins in leaves. However, acquired resistance caused by spraying the lower leaves with mercuric chloride instead of TMV was not accompanied with the appearance of new protein components [*25*]. Thus, the eventual rôle of the new protein components in resistance seems to be improbable.

Van der Plank [*24*] sees the mechanism of acquired resistance of plants to fungal infections in the increased level of phytoalexins after the first inoculation. Accumulation of phytoalexins and the resistance to a second (challenge) inoculation are local effects. In our system we have a case of resistance to a virus infection (in a local lesion host) where a plant hormone is involved in the mechanism. Both resistance and the stimulus (hormone) seem to be systemically distributed out of the primarily infected leaves, and at about the same time. The action of acquired resistance is against tissue necrotization (symptoms) and not against the virus *per se*. We suggested earlier [*7*] that necrotization caused by viruses can be regarded as an enhanced senescence effect. Cytokinin, as a juvenility hormone, acts against ageing; in other words, it acts against the development of disease symptoms. Zatykó [*27*] expressed the idea that a non-specific protective mechanism involving an increase in cytokinins may exist in plants against adverse effects. Compounds which increase senescence of plant tissues (e.g. ethylene and chloramphenicol) were antagonized in their action by the systemic acquired resistance. It is suggested that the increased cytokinin level may be involved in this effect.

NOTE ADDED IN PROOF

In a recent review paper by S. Gianinazzi & C. Martin on "Induced resistance and associated changes in protein metabolism in plants" which is in the press and will appear in a Symposium Volume on "*Current Topics in Plant Pathology*", edited by Z. Király, and published by Akadémiai Kiadó, the Publishing House of the Hungarian Academy of Sciences, Budapest, 1977, pp. 315–321, the idea is also expressed that different forms of induced resistance, including the systemic acquired resistance of tobacco to viruses, is accompanied by the production of new soluble proteins that are not present in control virus-free tobacco leaves.

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