THE EFFECT OF INFECTION BY TMV ON CYTOKININ LEVEL OF TOBACCO PLANTS, AND CYTOKININS IN TMV-RNA

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SUMMARY

The effect of virus infection on the cytokinin metabolism of host plants was investigated. Cytokinin activity was determined by applying the soybean callus bioassay. The results show, that the cytokinin activity in extracts prepared from leaves and stems of systemically infected tobacco plants (*N. tabacum* L. cv. Samsun) was much greater than that in extracts prepared from healthy plants.

In addition, cytokinin extracts were prepared from dark green and light green areas of systemically infected tobacco leaves, as well as from infected and healthy half-leaves of a local lesion host (*N. tabacum* L. cv. Xanthi-nc.). The extract of dark green areas had higher cytokinin activity than that of the extract of light green areas, and the infected Xanthi-nc half-leaves showed increased cytokinin activity compared with healthy half-leaves.

In order to study the reason for the increased cytokinin level in virus infected tissues, the possible presence of cytokinins in TMV itself was investigated. Our results show that hydrolysed TMV-RNA had some components with cytokinin activity, even if incorporated into the medium of soybean callus tissue culture without autoclaving. Although the cytokinins in TMV-RNA can not be responsible for the high cytokinin content of infected tissues, they can act as "triggers" in initiating metabolic and morphogenetic changes in virus infected plants.

INTRODUCTION

Numerous plant pathogens can induce growth and metabolic disturbances attributed to alterations in the levels or metabolism of plant hormones (cf. Sequiera, 1973; Balázs and Sziráki, 1974; Sziráki *et al.*, 1975). Several symptoms of virus diseases suggest that metabolism of growth regulators in the host cells is altered in some way. The endogenous levels of auxins, gibberellins and ethylene have been studied with virus diseases by many workers (cf. Andreae and Good, 1955; Balázs *et al.*, 1969; Bailiss, 1974).

In contrast to this, the changes of cytokinins in virus infected plants have hardly been investigated, despite the fact that certain symptoms (e.g. enations caused by different viruses, tumour production, increased metabolic activity) suggest that cytokinin level should be investigated.

Our laboratory has been interested in the determination of the effect of virus infection on cytokinin metabolism of host plants. The recent results of Sziráki and Gáborjányi (1974) showed that systemic infection by TMV can increase the cytokinin level of tobacco plants.

The present study was made to compare the cytokinin content in extracts prepared from dark green and light green areas of systemically infected tobacco leaves, as well as, in extracts made from infected and healthy half-leaves of local lesion host, and determine whether cytokinins are present in TMV particles.

MATERIALS AND METHODS

Extraction and bioassay of cytokinins

For extraction and purification of cytokinins, the procedures followed were as we described previously (Sziráki *et al.*, 1975). For further separation the cytokinin extracts were subjected to paper chromatography on Whatman No. 1 paper in different solvent systems. For cytokinin standards, zeatin (Z), zeatinriboside (ZR), and N^6- (Δ^2-isopentenyl)-adenine (2iP) were co-chromatographed. The chromatograms were divided into ten strips and each was tested for cytokinin activity on the basis of soybean callus bioassay.

Extraction and hydrolysis of TMV-RNA

The Ul strain of TMV was maintained in tobacco (*Nicotiana tabacum* L. cv. Samsun) and was extracted and purified using a modification of the procedures of Fraenkel-Conrat (1966), and Gooding and Hebert (1967). The phenol-procedure (Gierer and Schramm, 1956) was used for extraction of TMV-RNA from purified TMV.

After extraction, a sample of TMV-RNA was adjusted to 0.2 *N* $HClO_4$ and incubated for 45 min in pressure-cooker at 0.5 atm, or in water-bath at 90°C. The hydrolysate was adjusted to 7.8 pH and extracted three times with equal volumes of water-saturated *n*-butanol. The butanolic phase was evaporated to dryness, and all of the butanol was removed by repeated reevaporation with water. The residue was dissolved in ethanol and subjected to paper chromatography. After developing, the chromatograms were tested for cytokinin activity in the soybean callus bioassay.

RESULTS AND DISCUSSION

One month after systemic infection, cytokinin extracts were prepared from leaves and stems of *N. tabacum* cv. Samsun plants. The extracts were further separated by paper chromatography. The chromatograms were developed in a solvent: *tercier*-butanol-ammonia-water (3 : 1 : 1; v/v), and then tested for cytokinins in soybean callus bioassay. Cytokinin activity in extracts from virus-infected tobacco stems and leaves were found at R_F regions 0.8–1.0; 0.0–0.3 and 0.8–0.9 respectively. The total cytokinin activity (Fig. 1) in extracts from virus-infected plants is about twice as much as that in extracts prepared from healthy plants.

We determined the cytokinin content of extracts prepared from dark green and light green areas of systematically infected tobacco leaves. Fig. 2 shows the paper chromatographic separation of cytokinin activity of dark green and light green areas. Both extracts have two peaks of cytokinin activity, however, the activity in extracts

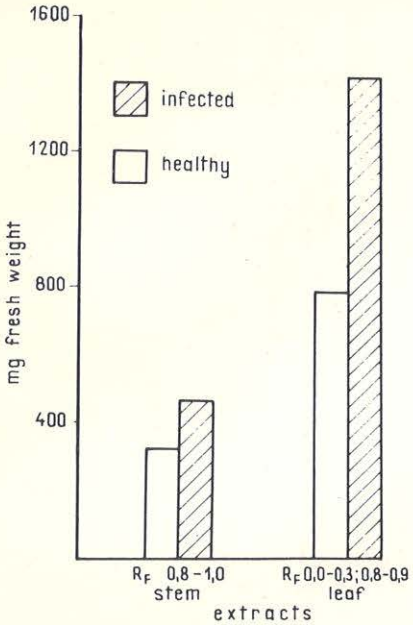

Fig. 1.

Total cell division activity in extracts prepared from healthy and systemically infected tobacco plants, after chromatography of the extracts in solvent: *tercier*-butanol-ammonia-water (3:1:1; v/v). The extracts were obtained from 50 g fresh weight of stems and leaves/1000 ml medium.
Ordinate: total callus yield (mg fresh weight); Abscissa: R_F values at which the extracts of stems and leaves respectively showed cell division activity

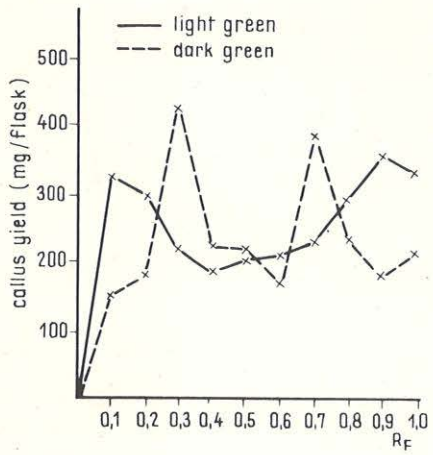

Fig. 2.

Paper chromatographic separation of cytokinin activity in extracts from light green and dark green areas of systemically infected tobacco leaves. The chromatograms were developed in ethyl acetate-formic acid-water (60:5:35; v/v, upper phase). The extracts were obtained from 55 g fresh weight of light green and dark green areas, respectively/1000 ml medium.
Ordinate: callus yield (mg/flask); Abscissa: R_F value

from dark green spots is slightly higher than that in extracts of light green spots. In addition, there are differences between the chromatographic migrations of active compounds obtained from light green areas and dark green areas. This suggests that chemically distinct factors are present in the two extracts, both at the low and at the high R_F regions.

The infection sites of several obligate parasites are often referred to as "metabolic sinks" (cf. Sequiera, 1973). Virus-inoculated local lesion hosts can produce spots with high radioactivity if they are exposed to $^{14}CO_2$ (Doke and Hirai, 1970).

Starting with these facts, we compared the cytokinin levels of healthy and of infected half-leaves of a local lesion host. The extracts were prepared 40 hrs after a heavy inoculation with TMV. The results reveal three peaks of cell division activity in the extract of healthy half-leaves, and four peaks in the extracts in the infected

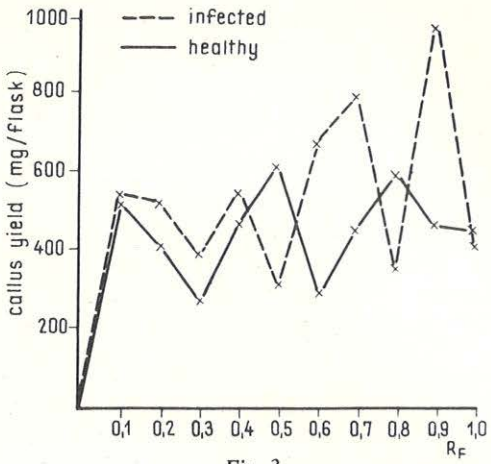

Fig. 3.

Soybean callus bioassay of extracts prepared from healthy and infected half-leaves of a local lesion host. The chromatograms were developed in ethyl acetate-formic acid-water (60 : 5 : 35; v/v, upper phase). The extracts were obtained from 55 g fresh weight of half-leaves/1000 ml medium. Ordinate: callus yield (mg/flask); Abscissa: R_F value

ones (Fig. 3). It can also be seen, that the total growth promoting activity is greater in extracts of infected half-leaves than in extracts of healthy half-leaves.

Increased levels of cytokinins have been frequently suggested to account for establishing a zone of intense metabolic activity around the infection sites of plant pathogens (cf. Goodman *et al.*, 1967; Sequiera, 1973). Plant viruses also can induce increases in protein and in RNA synthesis and in other metabolic processes (Goodman *et al.*, 1967; Doke and Hirai, 1970) in systemically infected plants. There are similar phenomena in the local lesion hosts too, at least under certain conditions (Doke and Hirai, 1970, Wu *et al.*, 1975).

Our data suggest, that the increased levels of cytokinins in a virus-infected systemic host, as well as in the infected half leaves of a local lesion host (before appearance of visible lesions) may result in inducing increased metabolic processes in virus-infected tissues.

Our further aim was to investigate the mechanism of the increased cytokinin level in virus-infected tissues. It is well known that natural cytokinins are present in plants in the free state, and as constituents of certain tRNA's (Miller, 1961;

Letham, 1963; Hall *et al.*, 1967), and they occur in specific tRNA's of other organisms (Hayashi *et al.*, 1969; Miller 1967; Robins *et al.*, 1967). It is interesting that several microorganisms, which can induce distorted growth of plants, secrete free cytokinins into the medium (Klämbt *et al.*, 1966; Phillips and Torrey, 1972).

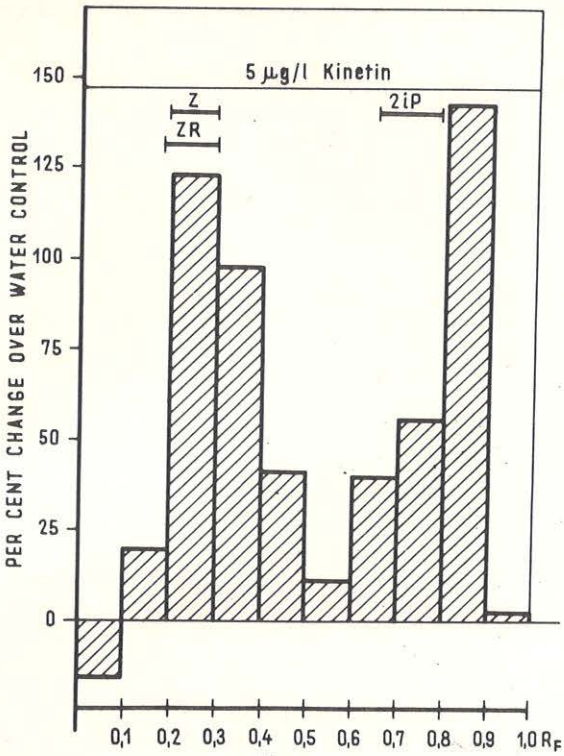

Fig. 4.

The paper chromatographic separation of cytokinin activity from butanolic extract of hydrolysed TMV-RNA. The hydrolysis was carried out at 0.5 atm. The chromatogram was developed in ethyl acetate-formic acid-water (60 : 5 : 35; v/v, upper phase).
Ordinate: per cent change of callus yield compared to water control; Abscissa: R_F value

In the course of our investigation we have demonstrated cytokinin activity in hydrolysed TMV-RNA, Table 1. and Fig. 4. show the results of bioassays of chromatograms of hydrolysed viral RNA. It can be seen from Table 1, that there are three peaks of cell division activity. By comparison with the standards, one can conclude that the activity at R_F 0.8–1.0 is due to a mixture of cytokinins containing Z, ZR and 2iP. The peaks at lower R_F regions do not correspond to the migration of any of our cytokinin standards. Fig. 4 also indicates, that the callus proliferation is due to Z, ZR and 2iP and presumably other cytokinins.

A question could arise whether the occurrence of the compounds with cytokinin activity in TMV-RNA is due to the high pressure applied during the handling. In order to eliminate this possibility we hydrolysed the TMV-RNA at normal pressure. The hydrolysate was filter-sterilized before adding to the medium of soybean tissue culture. The results reveal high cytokinin activity in TMV-RNA under these experimental conditions (Fig. 5).

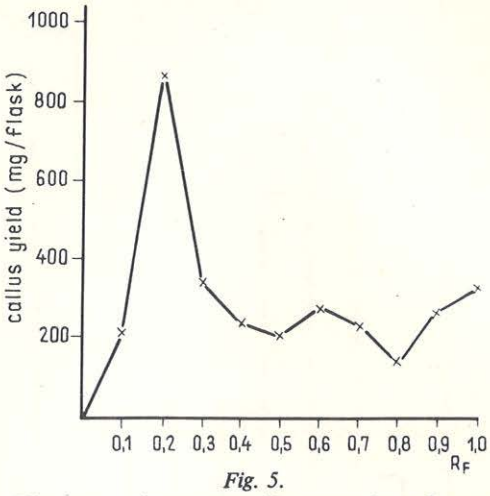

Fig. 5.

The paper chromatographic separation of cytokinin activity from butanolic extract of hydrolysed TMV-RNA. The hydrolysis was carried out at atmospheric pressure. The chromatogram was developed in ethyl acetate-formic acid-water (60 : 5 : 35; v/v, upper phase).
Ordinate: callus yield (mg/flask); Abscissa: R_F value

Table 1

Soybean callus bioassay of chromatogram of butanolic extract of hydrolysed TMV–RNA

R_F	0.0–0.1	0.1–0.2	0.2–0.3	0.3–0.4	0.4–0.5	0.5–0.6	0.6–0.7	0.7–0.8	0.8–0.9	0.9–1.0
Callus yield (mg/flask)	255	515	305	295	240	408	789	411	550	463
Kinetin controls										
Kinetin conc. (μg/liter)	0.0	5.0	15.0							
Callus yield (mg/flask)	192	282	406							

The extract was chromatographed on Whatman No. 1 paper in *t*-butanol – 25% NH_3 – water (3 : 1 : 1 v/v). The migration of Z, ZR and 2 iP standards corresponded to R_F regions: 0.81–0.86, 0,89–0.95 and 0.91–0.96 respectively. Dilution for this assay: 9.0 mg TMV–RNA/liter of test medium.

The TMV-RNA has been generally considered to contain no minor nucleotides, although Dunn and Smith (1959) found a trace of the nucleoside of 6-methyladenine.

It is also very interesting, that certain fragments of several plant and animal viruses can act as tRNA, at least in *in vitro* systems, and tRNA is the only RNA which has as yet been proved to contain natural cytokinins. The amino acid accepting fragment of TMV-RNA can be esterified with histidine by amino-acyl tRNA synthetase prepared from bean (Öberg and Philipson, 1972; Kohl and Hall, 1974).

Although the cytokinins in TMV-RNA can not be responsible for the high cytokinin content of infected tissues, they can act as "triggers" in initiating metabolic and morphogenetic changes in virus-infected plants.

We thank Dr. Lowell B. Johnson (Kansas State University) for sending the abstract of their paper presented on 67th Annual Meeting of The American Phytopathological Society, August 9–14 at Houston. The occurrence of 6-methyladenosine in TMV-RNA has been confirmed by their work, which demonstrated the presence of this minor base in RNA extracted from tobacco mosaic, cowpea mosaic, brome mosaic and cowpea chlorotic mottle viruses. Moreover, they found dihydrouridine and pseudouridine in RNA from cowpea chlorotic mottle virus and Belladonna mottle virus (Niblett *et al.*, 1976).

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