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Systemic infection of some N-gene-carrying Nicotiana species and cultivars after inoculation with tobacco mosaic virus

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Abstract

Since July 1974 *Nicotiana tabacum* 'Samsun NN' plants, inoculated with the common strain of tobacco mosaic virus (TMV), have occasionally been found to develop necrosis on non-inoculated upper leaves 2–7 days after the local necrotic lesions had appeared on the lower leaves. All these plants had been kept in a growth chamber at 17–20 °C. Other tobacco species and cultivars carrying the *N* gene, such as *N. glutinosa* and *N. tabacum* 'Xanthi-nc', showed the same phenomenon. Substantial amounts of TMV could be recovered from leaves with systemic symptoms. The systemic necrosis somewhat resembled that caused by tobacco rattle virus (TRV). A number of possible causes, such as high concentration of the inoculum, contamination with another strain of TMV or with TRV, change in the genetic composition of the host plants and certain growing conditions (soil, water, pesticides) were investigated. None of these factors could be held fully responsible for the abnormal systemic reaction, although there was evidence that the soil could sometimes play an important rôle.

Introduction

Tobacco species and cultivars carrying the *N* gene, such as *Nicotiana glutinosa*, *N. tabacum* 'Samsun NN' and *N. tabacum* 'Xanthi-nc', usually show only local necrotic lesions on the leaves after inoculation with tobacco mosaic virus (TMV). However, it is well-known that such tobacco plants, hypersensitive to infection with TMV, become systemically infected either by grafting on plants systemically infected with TMV (Köhler, 1941; Grison, 1961, 1968; Grison and Martin, 1961; Weintraub et al., 1961; Li and Schmelzer, 1964; De Leeuw, 1968) or by keeping the plants at elevated temperatures after inoculation. As the term 'elevated temperatures' is rather vague and the literature mentions a wide variation of temperatures all leading to systemic infection, we shall first give a survey of literature pertaining to the correlation between temperature and type of symptoms in tobacco species and cultivars carrying the *N* gene. Samuel (1931) found that *N. glutinosa* plants reacted to inoculation with TMV with small local lesions when kept at 21 °C after inoculation. The lesions increased in size when the temperature was raised to 28 °C, while at 35 °C there was no necrosis, the inoculated leaves showing faint yellow-blotch primary

lesions. This phenomenon was found to occur with all tobacco species and cultivars carrying the *N* gene (McKinney and Clayton, 1945). When after the heat treatment the temperature was lowered the plants became necrotic. Kassanis (1952) observed that *N. glutinosa* plants kept at 36°C after inoculation with TMV developed a variety of local symptoms: sometimes no lesions but a general yellowing of the inoculated leaves developed, at other times there were either chlorotic local lesions, with or without a necrotic ring, or concentric chlorotic rings and a line pattern. Symptoms of systemic infection without necrosis always appeared in young *N. glutinosa* plants two days after inoculation. When these systemically-infected plants were transferred to the glasshouse they became necrotic and collapsed in about one day. When TMV-inoculated *N. glutinosa* plants were exposed to high temperature after the local lesions had developed, the virus became systemic too. Martin and Gallet (1966a, b, c) observed systemic invasion by TMV of *N. tabacum* 'Xanthi-nc' plants when these were kept at 30°C after inoculation with TMV. The invaded tissues became necrotic when the plants were transferred back to 20°C. Shimomura (1972) did not find any movement of TMV from the inoculated leaves of *N. glutinosa* or 'Xanthi-nc' to the upper part of plants kept at 22°C. At 30°C, however, the plants became systemically invaded, developing symptoms on the top leaves one week after inoculation.

Holmes (1938) mentioned that the hybrid *N. tabacum* × *N. glutinosa* responds to infection with TMV as *N. glutinosa* does by the production of necrotic primary lesions. He further defined: 'In these lesions the virus is localized except in young plants where systemic necrosis frequently occurs'. In later publications Holmes (1954, 1960) stated that TMV in plants carrying the *N* gene tends to be restricted to the necrotic primary lesions except at high temperatures. He, however, added (1954): 'Occasionally virus may move along a midvein of a leaf to the stem and cause death of an individual plant, or, at the least, produce necrotic-type streaking of the stem by an extended primary lesion'.

Zaitlin (1962) observed the occasional systemic invasion of the hypersensitive TMV hosts *N. glutinosa* and 'Xanthi-nc' at an average mean daily high temperature of 25.5°C (the highest temperature recorded on any day was 29°C). This systemic invasion occurred after inoculation with two strains of TMV, viz. U_1 and U_2. Weintraub et al. (1963) reported, however, that they had never found such a systemic invasion of *N. glutinosa* with the common strain of TMV under *normal* conditions. They added: 'There is in the Vancouver laboratory, however, a strain of TMV, isolated by Dr. R. Stace-Smith and known as strain TMV-S (unpublished data) that appears to behave like Zaitlin's TMV strains U_1 and U_2. Under normal conditions TMV-S caused systemic necrosis in about 5% of *Nicotiana glutinosa* plants inoculated. Zaitlin's virus appears similar to TMV-S in this property, which suggests that the strain of virus may be of some consequence in this matter'. They further stated that temperatures around 28–30°C are rather critical in inducing marked changes of symptoms on TMV-inoculated leaves of *N. glutinosa*, as is our own experience.

Recently, Shimomura and Ohashi (1975) described necrotic lesions formed on non-inoculated upper leaves of *N. glutinosa* and 'Samsun NN' tobacco plants kept at 20°C for eight days after inoculation with TMV. Unable to detect TMV in leaves with these systemic lesions, they called them non-viral lesions.

Fig. 1. Non-inoculated top leaves of *Nicotiana tabacum* 'Samsun NN' with chlorotic spots, necrosis and malformation after inoculation of the lower leaves with TMV.

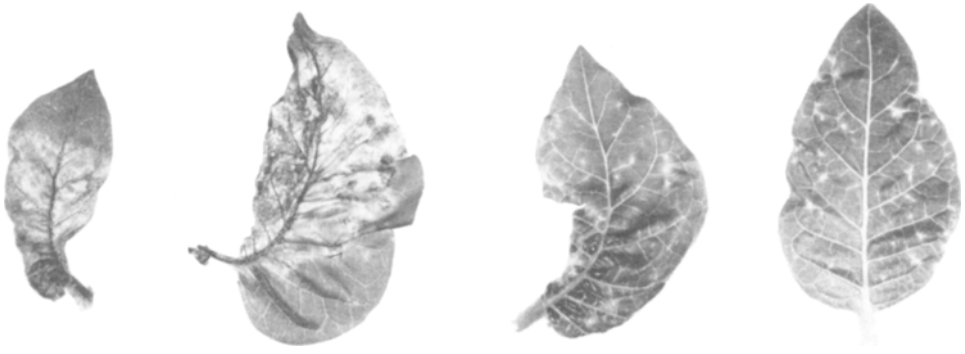

Fig. 1. Niet-geïnoculeerde topbladeren van Nicotiana tabacum 'Samsun NN' met chlorotische plekjes, necrose en misvorming na inoculatie van de onderste bladeren met TMV.

We have grown 'Samsun NN' plants in a growth chamber at 17–20 °C for 10 years and have never observed non-viral lesions (Van Loon, 1972). In July 1974 we discovered at Wageningen some 'Samsun NN' plants which showed chlorotic spots and necrosis on and malformation of the non-inoculated upper leaves about a week after necrotic lesions appeared on the lower leaves inoculated with the common strain of TMV (Fig. 1). In a couple of weeks these systemically reacting plants died after having shown severe top necrosis (Fig. 2). All plants had been kept in a growth chamber at 17–20 °C. A few months later the same phenomenon was observed in the glasshouse at Wageningen where nearly all TMV-inoculated 'Samsun NN' plants showed severe systemic necrosis. From that time onwards this unusual reaction of 'Samsun NN' tobacco to TMV has always occurred to a greater or lesser extent both in the growth chamber and in the glasshouse. In many respects the systemic symptoms resembled those caused by tobacco rattle virus (TRV).

Fig. 2. Plant of *Nicotiana tabacum* 'Samsun NN' showing severe top necrosis and malformation after inoculation of the lower leaves with TMV.

Fig. 2. Nicotiana tabacum 'Samsun NN'-plant met hevige topnecrose en misvorming na inoculatie van de onderste bladeren met TMV.

To explain this phenomenon the following hypotheses might be put forward:

1. High concentrations of TMV in the inoculum cause systemic spread (a 'dose effect').
2. There is a mutant of TMV in the inoculum.
3. Genetic changes in the tobacco species have taken place.
4. Besides TMV, TRV is present in the TMV-inoculated plants.
5. Certain growing conditions of the plants are responsible for the systemic spread.

In the present paper results of investigations to test these hypotheses are reported.

Materials and methods

Plants. At Wageningen, tobacco plants (*N. glutinosa*, *N. tabacum* 'Samsun NN', *N. tabacum* 'Xanthi-nc' and *N. tabacum* 'White Burley') were raised for 7–8 weeks in the Laboratory of Virology either in a glasshouse (henceforth referred to as glasshouse-Wageningen), where the temperature usually varied between 21 and 25°C, or in growth chambers at 17–20°C (henceforth referred to as 18°C) and 21–23°C (henceforth referred to as 22°C). Most of the seeds of the above tobacco species and cultivars originated from plants propagated by selfing in the glasshouse-Wageningen for more than 20 years. For comparison we also used seed of *N. glutinosa*, 'Samsun NN' and 'Xanthi-nc' recently obtained from Dr M. Zaitlin (Department of Plant Pathology, Cornell University, Ithaca, USA). Seeds were sown in pans filled with the commercial soil mixture Trio (Vriezeveen, Netherlands), commonly used in the glasshouse-Wageningen. It consists of peat, sand and fertilizer and is steam-sterilized before use. For comparison a commercial mixture (Jongkind Aalsmeer no. V/St. 400) consisting of peat, Finn peat, leaf-soil, stable manure, dune sand and fertilizer containing iron was also used.

In a few experiments, carried out in the Phytopathological Laboratory 'Willie Commelin Scholten', at Baarn, locally prepared steam-sterilized soil was used composed of 1 part compost, 1 part leaf mould, 1/4 part peat-dust and 1/10 part dried cow manure. After steaming, dolomite marl and Pokon were added.

Soon after emergence the seedlings were transferred to wooden (at Wageningen) or plastic (at Baarn) boxes and later to pots when they had attained a suitable size. Bean plants (*Phaseolus vulgaris* 'Bataaf') were grown in the glasshouse-Wageningen for 10 days after sowing. All the other test plants used were also raised in this glasshouse. Unless otherwise stated *N. glutinosa* and 'Samsun NN' plants were used in the 6-7-leaf-stage.

Viruses. TMV. In most experiments the TMV used was the common (Wageningen) strain, from now on designated as TMV-Wageningen. Purified preparations or crude sap from TMV-infected 'White Burley' plants were used as inocula. For comparison we also used TMV from a tobacco leaf obtained from Dr G. Melchers (Max-Planck-Institut für Biologie, Tübingen, F.R.G.). This TMV *vulgare* dated back to '*Nicotiana* virus I' received by Stanley in the thirties. Furthermore, the TMV-WU_1 strain originally isolated from TMV-Wageningen (Van Loon, 1972) was tested. In a few experiments the inoculum was a purified suspension of 10 μg/ml of the TMV isolate commonly used in the laboratory at Baarn. This TMV, however, had been obtained from the Laboratory of Virology at Wageningen in 1967. In some experi-

ments ground cigarettes of different brands were the source of TMV inoculum.

For TMV purification TMV-containing leaf material was homogenised in phosphate buffer (0.1 M, pH 7.0; w/v = 1/1) in a Waring blendor. After squeezing the pulp through cheese-cloth the sap was centrifuged at 8000 *g* for 15 min. To the supernatant polyethylene glycol 6000 was added to a concentration of 4% and NaCl to a concentration of 0.2 M. The mixture was stirred at room temperature for 1 h and centrifuged at 8000 *g* for 15 min. The pellet was resuspended in phosphate buffer containing 2% Triton X-100, and the suspension centrifuged at 8000 *g* for 15 min. The virus was further purified by two cycles of differential centrifugation at 78480 *g* for 1 h and 8000 *g* for 10 min and by rate zonal centrifugation. Sucrose density gradient columns (10–40% sucrose) were prepared in the phosphate buffer. After centrifugation (at 51500 *g* for 2.5 h) the tubes were monitored by passage through an LKB Uvicord analyser. The virus-containing fraction was dialysed against 0.1 M phosphate buffer for one night.

Purified preparations were negatively stained with 2% phosphotungstic acid, pH 6.5, and examined in a Siemens Elmiskop I electron microscope.

Preparations of TMV-RNA were obtained by the phenol method of Gierer and Schramm (1956).

TRV. Inocula of TRV were either crude sap from TRV-inoculated 'Samsun NN' plants or nucleic acid preparations obtained by extraction of TRV-infected 'Samsun NN' plants according to the phenol-bentonite method (Huttinga, 1972). Twenty grams of systemically-infected top leaves of 'Samsun NN' plants were homogenised in 20 ml of a glycine-NaOH buffer (0.1 M, pH 9.5, containing 0.1 M NaCl, 0.005 M Na_3EDTA, 1% sodium dodecylsulphate and 1% sodium bentonite) and 40 ml water-saturated phenol. After homogenisation in a Waring blendor at low speed the mixture was centrifuged at 3000 *g* for 5 min. The aqueous phase was collected and the phenol phase and interphase re-extracted with 20 ml buffer and centrifuged as described. The two aqueous phases were combined and extracted twice with an equal volume of water-saturated phenol. The aqueous phase was then freed from phenol by three extractions with diethylether. Excess ether was removed by passing nitrogen through the solution. Bentonite particles were removed by centrifuging at 6000 *g* for 10 min. After dialysing the solution against distilled water for 2 h, two volumes of cold ethanol were added and the mixture kept in a freezer. The precipitate formed was collected by centrifuging at 12000 *g* for 5 min and resuspended in 2.2 ml 0.01 M phosphate buffer pH 7.0, containing 0.005 M Na_3EDTA. For preparing the bentonite suspension 15 g of bentonite were suspended in 300 ml 0.01 M phosphate buffer pH 7.0, using a Waring blendor. After centrifugation at 2000 *g* the pellet was discarded and the supernatant centrifuged at 10000 *g*. The resulting pellet was resuspended in 0.01 M sodium phosphate buffer pH 7.0. This differential centrifugation was repeated twice. Finally the pellet was resuspended in the buffer to a concentration of 3–4% (w/v) as measured by determining the dry weight of 1 ml samples.

Further details will be given when describing the results.

Results

Reactions of N. glutinosa and 'Samsun NN' to inoculation with TMV. To compare *N. glutinosa* and 'Samsun NN' plants raised at 18°C and 22°C in their behaviour to inoculation with TMV, 10 plants of each of the two species at either temperature were inoculated with a pure suspension of TMV-Wageningen (10 μg/ml). After three days a comparable average number of local lesions on each or three leaves of both species appeared at 18°C (504 and 725, respectively). The lesions on the leaves of plants at 22°C were not counted, because they had already coalesced, but their number was comparable to that on the plants at 18°C. Within six days after inoculation all 'Samsun NN' plants at 22°C had developed systemic necrosis (but only one at 18°C), whereas none of the *N. glutinosa* plants reacted systemically, either at 18°C or at 22°C. However, at 10 days after inoculation, five of the *N. glutinosa* at 22°C showed clear systemic necrotic lesions and malformation on non-inoculated top leaves (Fig. 3), and three of the *N. glutinosa* plants at 18°C had small chlorotic spots on the non-inoculated leaves (Fig. 4). By that time six of the 'Samsun NN' plants at 18° showed severe, systemic symptoms.

The incidence of systemic necrosis decreased sharply with the age of the plants. In some cases 'Samsun NN' plants with severe top necrosis showed regeneration with healthy-looking shoots developing from axillary buds (Fig. 5). Bio-assay did not reveal the presence of virus in these shoots. Sometimes such shoots showed systemic necrosis too.

The presence of TMV in systemically-infected leaves of N. glutinosa and 'Samsun NN'. After mechanical transmission. From TMV-inoculated, systemically-reacting *N. glutinosa* plants both top leaves with severe necrosis, chlorosis and malformation (T_G) and inoculated leaves with local lesions (I_G) were ground separately in distilled water (3 g leaf tissue in 8 ml water) and the sap was inoculated onto 20 detached *N. glutinosa* leaves. Sap from T_G yielded an average number of 198 local lesions per leaf, that from I_G (by that time senescent and desiccated) 9.

Fig. 3. Non-inoculated top leaves of *Nicotiana glutinosa* with necrotic lesions and malformation after inoculation of the lower leaves with TMV.

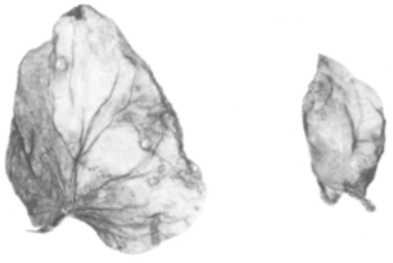

Fig. 3. Niet-geïnoculeerde topbladeren van Nicotiana glutinosa met necrotische lesies en misvorming na inoculatie van de onderste bladeren met TMV.

Fig. 4. Non-inoculated top leaves of *Nicotiana glutinosa* with chlorotic spots after inoculation of the lower leaves with TMV.

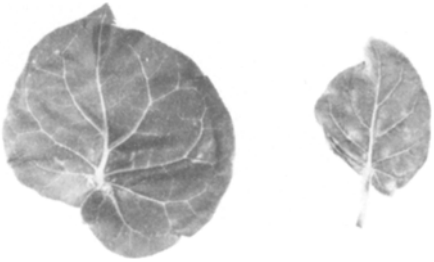

Fig. 4. Niet-geïnoculeerde topbladeren van Nicotiana glutinosa met chlorotische plekjes na inoculatie van de onderste bladeren met TMV.

Fig. 5. Plant of *Nicotiana tabacum* 'Samsun NN' with a vigorously growing, healthy-looking lateral shoot on the main stem of which the top (arrow) had died.

Fig. 5. Plant van Nicotiana tabacum 'Samsun NN' met een krachtig groeiende, gezond-uitziende zijscheut aan de hoofdstengel, waarvan de top (pijl) afgestorven is.

From TMV-inoculated 'Samsun NN' plants both top leaves showing necrosis and malformation (T_S) and inoculated leaves with local lesions (I_S) were ground separately in distilled water (3.3 g leaf tissue in 5 ml water) and the sap was inoculated onto 20 detached *N. glutinosa* leaves. Sap from T_S yielded an average number of 158 local lesions per leaf, that from I_S 389.

When sap from T_S and I_S was inoculated onto three plants each of *N. glutinosa* and 'Samsun NN' in the glasshouse-Wageningen, all plants of both species showed systemic necrosis four days after inoculation with sap from T_S, whereas only one 'Samsun NN' plant and none of the *N. glutinosa* plants was systemically infected after inoculation with sap from I_S at that time. No more plants developed systemic necrosis at later dates. In this experiment the average number of the resulting local lesions on *N. glutinosa* were 74 (T_S) and 40 (I_S), those on 'Samsun NN' 72 (T_S) and 78 (I_S).

When sap from T_S was inoculated onto 10 'Samsun NN' plants in growth chambers all plants showed systemic symptoms at five days after inoculation, both at 18°C and at 22°C. 'Samsun NN' plants inoculated for comparison with a pure suspension of TMV-Wageningen (10 μg/ml) also showed systemic symptoms at five days after inoculation, but in fewer plants at 18°C (4/10) than at 22°C (10/10). At 18°C, the average number of local lesions on each of three leaves of 'Samsun NN' plants inoculated with either T_S or purified TMV suspension was 707 and 453, respectively.

Electron microscopy of dip preparations of T_S revealed the presence of broken TMV-like particles (Fig. 6). In dip preparations of I_S, on an average only one virus particle per field of vision was visible.

To find out whether TMV in T_S differs from that in I_S the virus from both was purified 3–4 weeks after inoculation. From 200 g T_S 0.07 mg TMV was obtained;

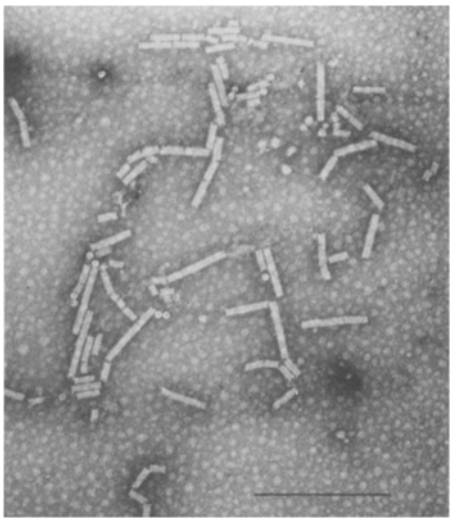

Fig. 6. Electron micrograph of a dip preparation of TMV from a systemically infected top leaf of *Nicotiana tabacum* 'Samsun NN'. Bar represents 300 nm.

Fig. 6. Elektronenmicroscopische foto van een indooppreparaat van TMV uit een systemisch-geïnfecteerd topblad van Nicotiana tabacum 'Samsun NN'. Het vergrotingsstreepje geeft 300 nm weer.

from a similar amount of I_S 0.3 mg TMV. Both the purified TMV suspensions showed the usual UV absorption profile. In the electron microscope, both the T_S suspension and the I_S suspension showed many disrupted virus particles (Fig. 7A and B). Assay of both purified suspensions at 25 μg/ml on 10 detached *N. glutinosa* leaves yielded 54 local lesions per leaf for the suspension from T_S and 53 for that from I_S. Both purified suspensions (at 5 μg/ml) inoculated onto 15 'Samsun NN' plants yielded five systemically infected plants for T_S and six plants with systemic symptoms for I_S at 18 °C 10 days after inoculation. No TMV could be demonstrated by bio-assay in symptomless non-inoculated leaves of *N. glutinosa* or 'Samsun NN' plants.

Summarizing we may say that TMV was present in T_S. Crude sap from T_S induced more systemic infection than sap from I_S, whereas inoculation with purified suspensions of T_S and I_S yielded a comparable number of systemically infected plants.

After grafting. When 20 'Samsun NN' and 20 *N. glutinosa* plants were grafted on 'Samsun NN' or *N. glutinosa* plants and the rootstocks inoculated with a pure TMV-Wageningen suspension of 40 μg/ml on the 14th day after grafting, systemic necrosis developed in the scions of both species about five weeks after grafting on 'Samsun NN' (Table 1). When *N. glutinosa* acted as rootstocks only doubtful symptoms developed on the scions.

Hypothesis 1: Concentration of inoculum. To investigate a possible effect of TMV concentration in the inoculum on the appearance of systemic symptoms in 'Samsun NN', a series of 10-fold dilutions, ranging from 1000 to 0.01 μg/ml was inoculated onto five 'Samsun NN' plants per dilution, at 18 °C. At 1000, 100 and 10 μg/ml all five plants showed systemic necrosis. With 1 μg/ml three out of the five plants reacted systemically; with 0.1 μg/ml and 0.01 μg/ml no plant showed a systemic reaction, although in the former case on an average about 60 local lesions per leaf had appeared. The systemic symptoms increased in severity with higher doses.

Fig. 7. Electron micrographs of TMV suspensions purified from systemically-infected top leaves (A) and inoculated leaves (B) of *Nicotiana tabacum* 'Samsun NN'. Bar represents 300 nm.

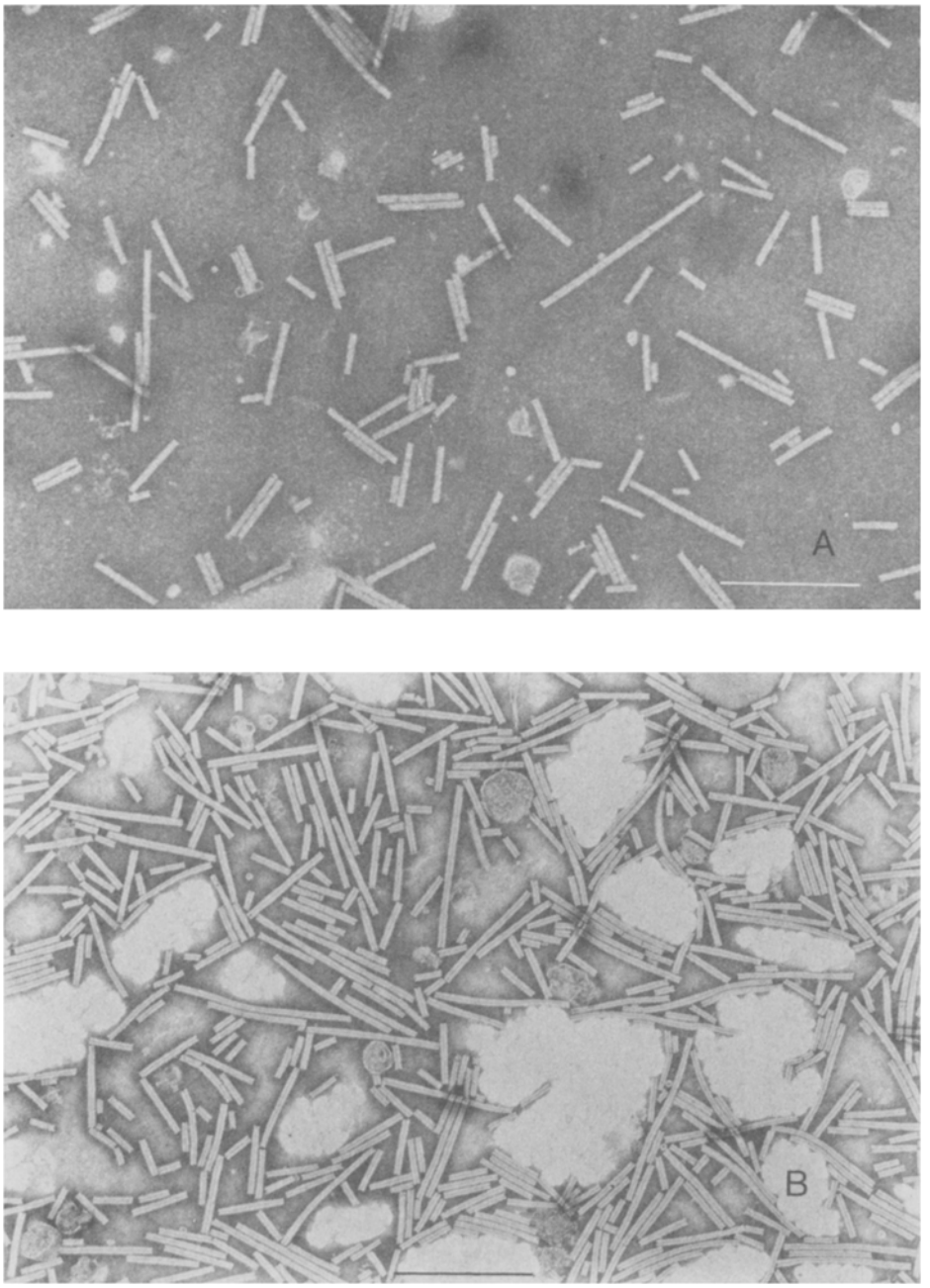

Fig. 7. Elektronenmicroscopische foto's van TMV-suspensies, gezuiverd uit systemisch-geïnfecteerde topbladeren (A) en geïnoculeerde bladeren (B) van Nicotiana tabacum 'Samsun NN'. Het vergrotingsstreepje geeft 300 nm weer.

Table 1. Necrosis in the scions of tobacco plants after inoculation of their rootstocks with TMV.

Scion/rootstock combination	Number of scions		
	with necrosis	without necrosis	with doubtful symptoms
SNN*/SNN	4	12	1
SNN/G1**	0	13	0
G1/G1	0	4	6
G1/SNN	9	0	4

* *Nicotiana tabacum* 'Samsun NN'; ** *Nicotiana glutinosa.*

Tabel 1. Necrose in de enten van tabaksplanten na inoculatie van hun onderstammen met TMV.

Hypothesis 2: Possible presence of a mutant of TMV. Single lesion transfer. To search for a TMV mutant local lesions on leaves of *N. glutinosa* plants inoculated with a pure TMV-Wageningen suspension of 0.05 μg/ml yielding about five lesions per leaf were punched out and subinoculated onto *N. glutinosa* leaves through five successive passages. Eventually, sap from each of 18 lesions was separately inoculated onto one 'White Burley' plant for propagation. After about 14 days one mosaic-showing leaf from each 'White Burley' plant was separately ground in distilled water and the sap inoculated onto three 'Samsun NN' plants. No difference in reaction could be observed among the 'Samsun NN' plants inoculated with different isolates; in each of the 18 series one or more 'Samsun NN' plants showed systemic necrosis.

Virus from different sources. Another way to find out whether the systemic necrotic reaction might be due to the presence of a mutant was to test TMV from different origins. To this end TMV present in cigarettes and a cigar of the following brands, manufactured in different parts of the world, was investigated: Bisonte (Spain), Caballero (Holland), Chief Whip (Holland), Christo Cassimis (A.R.E.), Escort (Indonesia), Ganesh beedi (India), Gauloise (France), Kretek, a native cigarette with clove favour (Indonesia), North State (Holland) and a cigar from Havana (Cuba). Each sample of dry tobacco was soaked for about 10 min in a few ml's of phosphate buffer (pH 7), then ground in a mortar and the suspension obtained inoculated onto one 'White Burley' plant for propagation. The isolate from Caballero cigarettes had previously been propagated in 'Samsun' plants and was put at our disposal by Ir. C. P. de Jager (Laboratory of Virology, Wageningen). In all cases but one, the 'White Burley' plants reacted with more or less severe mosaic, comparable to that by TMV-Wageningen. Only the Caballero isolate, produced local lesions and no mosaic on 'White Burley'.

Symptom-showing leaves of each of these 'White Burley' plants were ground separately and the sap inoculated onto 15 'Samsun NN' plants each. In all but two groups of 'Samsun NN' plants, kept at 18°C, systemic necrosis appeared (Table 2). Only the isolates from Escort and Caballero gave local lesions only. Caballero, however, had already been found exceptional in causing only local lesions on 'White Burley' tobacco. Because of its exceptional behaviour the Escort isolate was tested again. This time systemic infection of 'Samsun NN' occurred.

Besides TMV from cigarettes and a cigar, isolates from Tübingen and Baarn were

Table 2. Systemic reaction in *Nicotiana tabacum* 'Samsun NN' inoculated with TMV originating from tobacco in cigarettes and a cigar.

Brand of cigarette and cigar	Number of plants with systemic reaction over number of inoculated plants
Bisonte	6/14
Caballero	0/15
Chief Whip	8/15
Christo Cassimis	9/14
Escort Exp. 1	0/15
Exp. 2	6/10
Ganesh beedi	6/15
Gauloise	4/14
Kretek	7/13
North State	4/15
Havana Cigar	6/15

Tabel 2. Systemische reactie in Nicotiana tabacum 'Samsun NN' geïnoculeerd met TMV afkomstig van tabak uit sigaretten en een sigaar.

tested on 'Samsun NN' plants, as well as the TMV-WU_1 strain. In all three cases systemic necrotic reactions were observed to the same extent as with TMV-Wageningen which gave comparable numbers of local lesions at the concentrations used (for TMV-Baarn and TMV-Wageningen 5 μg/ml). Table 3 shows that at 22 °C the isolate from Baarn caused visible systemic necrosis as early as five days after inoculation, whereas TMV-Wageningen did so in six days. In both series, at 18 °C the symptoms were less severe than at 22 °C; instead of necrotic lesions, top necrosis and severe malformation, a few chlorotic spots, sometimes with a light brown necrotic centre, were visible on the non-inoculated leaves.

Summarizing we may say that TMV from all the isolates tested gave rise to systemic infection.

Table 3. Systemic reaction in plants of *Nicotiana tabacum* 'Samsun NN' inoculated with purified TMV suspensions from Baarn and Wageningen and kept at 18 °C and 22 °C.

Days after inoculation	Baarn		Wageningen	
	18 °C	22 °C	18 °C	22 °C
5	0/10*	2/10	0/10	0/10
6	0/10	8/10	0/10	8/10
10	1/10	10/10	1/10	10/10
25	3/10	10/10	2/10	10/10

* Number of plants showing systemic reaction over number of inoculated plants.

Tabel 3. Systemische reactie in planten van Nicotiana tabacum 'Samsun NN' geïnoculeerd met gezuiverde TMV-suspensies uit Baarn en Wageningen en gezet bij 18 °C en 22 °C.

Table 4. Systemic reaction to TMV in tobacco species and cultivars raised from seed from USA.

Tobacco species and cultivars	Number of plants showing systemic reaction over number of inoculated plants
N. glutinosa	3/20
'Samsun NN' (glasshouse)	8/19
'Samsun NN' (growth chamber)	3/20
'Xanthi-nc'	3/20

Tabel 4. Systemische reactie op TMV bij tabakssoorten en -cultivars gegroeid uit zaad afkomstig uit de VS.

Hypothesis 3: Genetic changes in tobacco species. Although it seemed unlikely that different *Nicotiana* species would have simultaneously undergone an identical change in their genetic potential, seeds of different origins and from different batches of the same origin were compared. For this purpose we used seed of *N. glutinosa*, 'Samsun NN' and 'Xanthi-nc' from USA. The plants were raised in the glasshouse-Wageningen and shortly before inoculation the 'Samsun NN' plants were divided into two lots: one remained in the glasshouse, the other was transferred to a growth chamber at 18 °C. Twenty plants of each species or cultivar were inoculated with a pure suspension of TMV-Wageningen of 5 μg/ml. In all series systemically infected plants occurred (Table 4).

Furthermore, 'Samsun NN' seed harvested in the glasshouse-Wageningen in 1964, 1968 and 1971 was also used. Twenty plants raised from seeds of each series were inoculated with a pure TMV-Wageningen suspension of 5 μg/ml (10 μg/ml in case of the 1964 series) and kept in the glasshouse. Ten days after inoculation a number of plants from each series showed systemic necrosis (1964: 14/20; 1968: 13/20; 1971: 14/20).

Hypothesis 4: The presence of TRV. As the systemic necrosis in 'Samsun NN' with TMV resembled that caused by TRV, a possible contamination was investigated. So far, electron microscopy of purified preparations from T_S had yielded only TMV-like particles, but small numbers of TRV particles might have been overlooked. As bio-assay usually is the most sensitive method to detect small amounts of virus we selected a range of species and cultivars known to react differentially to TMV and TRV. Three plants of each species or cultivar were inoculated with the following inocula: TRV-containing tobacco sap, sap from T_S, and a purified TMV-Wageningen suspension of 50 μg/ml, respectively. Of a control series two plants were inoculated with sap from healthy-looking, uninoculated 'Samsun NN' plants and one remained uninoculated. The following plant species and cultivars were used: *Beta vulgaris*, *Capsicum frutescens*, *Chenopodium amaranticolor*, *Cucumis sativus*, *Fagopyrum esculentum*, *Gomphrena globosa*, *Hyoscyamus niger*, *Lycopersicum esculentum*, *Nicotiana clevelandii*, *N. glutinosa*, *N. rustica*, *N. tabacum* 'Bright Yellow', 'Samsun NN', 'Xanthi-nc', 'White Burley', *Petunia hybrida*, *Phaseolus vulgaris* 'Bataaf', *Physalis floridana*, *Pisum sativum* 'Mansholt', *Sinapis alba*, *Solanum melongena*, *S. tuberosum*, *Sonchus asper*, *Spinacia oleracea*, *Tetragonia tetragonoides*, *Vicia faba*, *Vigna sinensis* and *Zinnia elegans*. All plants inoculated with T_S and TMV showed

Table 5. Reaction of 'Samsun NN' and bean plants to nucleic acid extracts from upper leaves (T_S) and roots (R_S) of 'Samsun NN' systemically infected with TMV, from leaves of 'Samsun NN' infected with tobacco rattle virus (TRV) and from a purified TMV suspension (TMV-RNA).

Inoculum	Assay plants	
	'Samsun NN'	bean 'Bataaf'
T_S	LL*, S 1/8**	–
R_S	LL, S 2/8	–
TRV	LL, S 8/8	LL
TMV-RNA	LL, S 5/8	–

*Local lesions; **Number of plants showing systemic (S) symptoms over number of inoculated plants.

Tabel 5. Reactie van 'Samsun NN' en boneplanten op nucleïnezuurextracten uit de bovenste bladeren (T_S) en wortels (R_S) van 'Samsun NN' systemisch geïnfecteerd met TMV, uit bladeren van 'Samsun NN' geïnfecteerd met tabaksratelvirus (TRV) en uit een gezuiverde TMV-suspensie (TMV-RNA).

typical TMV symptoms; none of them showed symptoms which could be attributed to TRV. No symptoms appeared on the plants of the control series.

To find out whether uncoated TRV-RNA was present in T_S and roots (R_S) of 'Samsun NN' plants systemically infected after inoculation with TMV, nucleic acid from leaves and roots was extracted according to the phenol-bentonite method (Huttinga, 1972). Eight plants of 'Samsun NN' and bean 'Bataaf' were inoculated with each of these extracts. For comparison extracts from leaves of 'Samsun NN' plants systemically infected with TRV as well as TMV-RNA solutions were inoculated. Table 5 shows that bean reacted positively to TRV only. In all four series 'Samsun NN' reacted with systemic necrosis, but the symptoms by TRV were clearly different (milder, little malformation) from those caused by the other three inocula (Fig. 8A and B).

Hypothesis 5: Growing conditions of the plants. To see whether there is an effect of environmental conditions three factors have been studied, viz. soil, water and pesticides.

Soil. 'Samsun NN' plants from Wageningen seed were raised in soil from different origins. Some of the experiments were carried out at Wageningen (with Trio and Jongkind soils), others at Baarn (with the locally prepared soil only). The habit of the plants raised in the soil from Baarn differed considerably from that of the plants in the soils used at Wageningen. The leaf colour of the Baarn plants was lighter and the stems had much longer internodes. Because of their height they looked older although they were the same age as the plants at Wageningen, and the number of fully-developed leaves was also the same. The plants at Wageningen were inoculated with purified TMV suspensions both from Wageningen and Baarn, those at Baarn with the local isolate only. All the experiments carried out at Wageningen yielded plants systemically infected about 10 days after inoculation, irrespective of the type of soil used. The plants at Baarn, however, kept there in the glasshouse at temperatures comparable to those at Wageningen (20–22 °C), showed no systemic symptoms even 14 days after inoculation. We then transferred four pots with plants from

Fig. 8. Systemically infected top leaves (T) and inoculated lower leaves (I) of *Nicotiana tabacum* 'Samsun NN' after inoculation with tobacco rattle virus (A) and with TMV (B). Note the slightly lop-sided leaf at T with some chlorotic spots (A), and the clearly malformed leaves at T with chlorotic and necrotic spots (B).

Fig. 8. Systemisch geïnfecteerde topbladeren (T) en geïnoculeerde onderste bladeren (I) van Nicotiana tabacum 'Samsun NN' na inoculatie met tabaksratelvirus (A) en TMV (B). Let op het enigszins scheefgetrokken blad bij T met chlorotische plekjes (A) en de duidelijk misvormde bladeren bij T met chlorotische en necrotische plekjes (B).

Baarn to a growth chamber (22°C) at Wageningen. Three days after transfer, 1 out of the 4 plants showed a very clear systemic necrosis. Fourteen days after transfer, a second plant showed systemic necrotic lesions on two top leaves. By that time at Baarn 1 out of 20 plants also showed systemic necrosis and malformation. Top leaves of this plant were ground in distilled water and the sap was inoculated onto three 'Xanthi-nc' and three 'Samsun NN' plants. All inoculated leaves showed local lesions, but no systemic reaction was observed, even three weeks after inoculation. We then examined the two plants at Wageningen still without systemic symptoms for TMV in their top leaves. Three such leaves of each of the two plants were ground in 2 ml distilled water and the sap separately inoculated onto 20 detached *N. glutinosa* leaves. After five days there were no local lesions.

At Baarn a new series of 25 'Samsun NN' plants was inoculated with a purified suspension of 15 μg/ml TMV. Eleven days after inoculation 4 out of 25 plants showed systemic necrosis in the glasshouse where on sunny days the temperature rose from 24°C to 26°C. For comparison, 89 healthy, inoculable 'Samsun NN' plants in pots were transferred from Baarn to Wageningen, where they were distributed over the growth chambers at 18°C and 22°C (16 and 26 plants, respectively) and two glasshouses (20 and 27 plants, respectively). All plants were inoculated with a purified TMV-Wageningen suspension of 5 μg/ml. Eight days after inoculation 4 out of 16 plants at 18°C and 13 out of 26 plants at 22°C showed systemic necrosis. In the glasshouses 1 out of 20 and 3 out of 27 plants, respectively, showed systemic necrosis about 14 days after inoculation. This shows that the frequency of systemic infection is much lower with soil from Baarn than with that from Wageningen, even when the experiments were carried out at the latter place.

Water. As the source of water in the glasshouse-Wageningen and in the growth

chambers is different (the former being a well next to the glasshouse, the latter the municipal water supply) plants raised in the glasshouse and those in the growth chambers already received different types of water. Therefore, it was not necessary to carry out experiments with water from still another source.

Pesticides. A possible effect of pesticides was investigated by raising 50 'Samsun NN' plants in the glasshouse-Wageningen without any spraying with pesticides. They were inoculated with a purified TMV-Wageningen suspension of 5 μg/ml. Also after inoculation no pesticides had been applied. For comparison a similar number of 'Samsun NN' plants raised in the glasshouse-Wageningen and regularly sprayed with insecticides as a normal routine was also inoculated. After about 10 days a comparable number of plants in both series showed systemic symptoms.

Discussion

From the results it is clear that the systemic necrotic reaction to TMV we have observed during the last couple of years in *N*-gene-carrying tobacco species and cultivars cannot be attributed to the loss of the property of reacting with local necrotic lesions due to elevated temperatures. However, the effect of temperature on the occurrence of systemic necrosis was not negligible, as the systemic reaction was more frequent at 22 °C than at 18 °C.

Although the systemic symptoms in *N. glutinosa* and 'Samsun NN' resemble those described by Shimomura and Ohashi (1975), they differ from the non-viral lesions in that a substantial amount of TMV could be recovered from leaves showing these systemic symptoms. Similarly, the presence of TMV in *N. glutinosa* and 'Samsun NN' plants showing necrosis after grafting on TMV-infected 'Samsun NN' plants could easily be demonstrated.

The dose effect was not considered to be a major factor in overcoming hypersensitivity as there is no proportionality between the number of local lesions and systemic necrosis in 'Samsun NN', as shown in the experiments on mechanical transmission and dilution.

The possibility that in the course of time our common strain of TMV had become contaminated with another strain of TMV is unlikely as isolates of TMV from different places reacted similarly.

A change in the genetic composition of our *N. glutinosa* and 'Samsun NN' plants could also be ruled out as plants from seed of different origin or years of harvest showed the same phenomenon.

No TRV could be demonstrated in the systemically infected top leaves of 'Samsun NN' plants after inoculation with TMV, showing that the inoculum was not contaminated with TRV.

Judging from the differences in incidence of systemic necrosis observed between plants raised at Baarn and at Wageningen, growing conditions of the plants seem to play an important rôle, although by themselves they may not be responsible for the changed TMV-hypersensitive host relationship.

The above results do not exclude the possibility that besides TMV, some other factor be present especially in the top leaves. An indication in this direction is the fact that unpurified sap from top leaves of systemically infected 'Samsun NN' plants inoculated onto 'Samsun NN' yielded more (and quicker) systemically infected

plants than sap from leaves with local lesions or a purified TMV suspension of comparable infectivity.

Samenvatting

Systemische infectie van enkele N-gen-bevattende Nicotiana-soorten en -cultivars na inoculatie met tabaksmozaïekvirus

Tabakssoorten en cultivars die het *N* gen bevatten, zoals *Nicotiana glutinosa*, *N. tabacum* 'Samsun NN' en *N. tabacum* 'Xanthi-nc' vertonen gewoonlijk slechts lokale, necrotische lesies op de bladeren na inoculatie met tabaksmozaïekvirus (TMV).

In 1975 hebben Shimomura en Ohashi vermeld, dat necrotische lesies ontstonden op de niet-geïnoculeerde bovenste bladeren van planten van *N. glutinosa* en 'Samsun NN', die acht dagen na inoculatie met TMV bij 20 °C hadden gestaan. Daar ze in deze bovenste bladeren geen TMV konden aantonen, spraken deze auteurs van 'non-viral lesions'. Al gedurende meer dan 10 jaar zijn in Wageningen 'Samsun NN'-planten in een klimaatkamer bij 17–20 °C gekweekt maar nooit werden dergelijke 'non-viral lesions' waargenomen.

In juli 1974 werden in Wageningen enkele 'Samsun NN'-planten gevonden met chlorotische vlekjes, necrose op, en misvorming van, de bovenste bladeren, ongeveer een week nadat de lokale necrotische lesies waren verschenen op de met TMV geïnoculeerde onderste bladeren (Fig. 1). In een paar weken tijd stierven de meeste van deze systemisch geïnfecteerde planten na sterke topnecrose af (Fig. 2). Al deze planten bevonden zich in een klimaatkamer bij temperaturen die varieerden van 17–20 °C. Later werd een zelfde verschijnsel ook in een kas in Wageningen waargenomen. Van die tijd af is deze ongewone reactie van 'Samsun NN' op TMV altijd in meer of minder heftige mate opgetreden, zowel in de klimaatkamer als in de kas. In veel opzichten doen de systemische symptomen op 'Samsun NN' denken aan die, welke door tabaksratelvirus (TRV) op deze cultivar worden veroorzaakt.

Om dit verschijnsel te verklaren zijn de volgende hypotheses opgesteld:

1. Hoge concentraties van TMV in het inoculum veroorzaken systemische verspreiding (zg. dosis-effect).
2. Er is sprake van een mutant van TMV in het inoculum.
3. In de tabakssoorten zijn genetische veranderingen opgetreden.
4. Naast TMV is TRV aanwezig in de met TMV geïnoculeerde planten.
5. Bepaalde groeiomstandigheden van de planten zijn verantwoordelijk voor de systemische verspreiding.

Eerst zijn de reacties van *N. glutinosa* en 'Samsun NN'-planten op inoculatie met een zuivere suspensie van TMV-Wageningen vergeleken bij 18 °C en 22 °C. Zes dagen na inoculatie vertoonden de eerste 'Samsun NN'-planten systemische necrose, en na 10 dagen verschenen op enkele *N. glutinosa*-planten uit de serie van 22 °C duidelijke, systemische, necrotische lesies en misvormingen (Fig. 3) en bij 18 °C kleine, chlorotische vlekjes op de niet-geïnoculeerde bladeren (Fig. 4).

In enkele gevallen vertoonden 'Samsun NN'-planten met hevige topnecrose regeneratie doordat nieuwe, gezond-uitziende scheuten zich uit okselknoppen ontwikkeld hadden, nadat de top van de hoofdstengel was afgestorven (Fig. 5).

In de topbladeren van systemisch-reagerende *N. glutinosa* en 'Samsun NN'-plan-

ten kon TMV worden aangetoond; sap uit laatstgenoemde topbladeren (T_S), geïnoculeerd op jonge 'Samsun NN'-planten gaf meer systemische infectie dan sap uit bladeren van 'Samsun NN'-planten met lokale lesies (I_S).

Indooppreparaten van T_S gaven in de elektronenmicroscoop gebroken, TMV-achtige deeltjes te zien (Fig. 6).

Om te zien of het virus in de topbladeren verschilt van dat in de geïnoculeerde bladeren werd TMV gezuiverd uit T_S en I_S. Beide gezuiverde TMV-suspensies vertoonden het gewone UV-absorptie profiel en gaven in de elektronenmicroscoop veel gebroken virusdeeltjes te zien (Fig. 7A en B). De infectiositeit van beide gezuiverde suspensies, getoetst op afgeknipte bladeren van *N. glutinosa* en hele planten van 'Samsun NN' was gelijk.

Bij enting van 'Samsun NN' en *N. glutinosa*-planten op 'Samsun NN' of *N. glutinosa* en inoculatie van de onderstammen met TMV-Wageningen ontwikkelde zich systemische necrose na enting op 'Samsun NN' (Tabel 1).

Hypothese 1: *Dosiseffect.* Een serie van tienvoudige verdunningen met concentraties variërend van 1000 tot 0,01 µg/ml werd geïnoculeerd op 'Samsun NN'-planten. Bij de drie hoogste concentraties vertoonden alle planten systemische necrose, bij de twee laagste gaf geen der planten een systemische reactie.

Hypothese 2: De aanwezigheid van een mutant van TMV. Achttien lokale-lesie isolaten, verkregen na vijf passages op *N. glutinosa*, gaven alle systemische infectie van 'Samsun NN'-planten.

Tevens werd gewerkt met virus van verschillende herkomst. Tabakssap van negen merken sigaretten en een sigaar werd voor dit doel gebruikt. In acht van de negen series 'Samsun NN'-planten verscheen systemische necrose (Tabel 2). Bij 'Samsun NN'-planten geïnoculeerd met TMV-isolaten uit Tübingen, Baarn en Wageningen (Tabel 3) werden eveneens systemische reacties waargenomen.

Hypothese 3: *Genetische verandering in tabakssoorten.* Tabakszaad van verschillende herkomst, of van verschillende partijen van dezelfde herkomst, werd vergeleken. Planten van *N. glutinosa*, 'Samsun NN' en 'Xanthi-nc' uit de V.S. werden geïnoculeerd met een gezuiverde suspensie van TMV-Wageningen. In alle series verschenen systemisch-geïnfecteerde planten (Tabel 4). Bovendien werd 'Samsun NN'-zaad dat in verschillende jaren (1964, 1968, 1971) was geoogst in de kas in Wageningen, getoetst. In alle drie series trad systemische necrose op.

Hypothese 4: De aanwezigheid van TRV. Sap uit T_S werd geïnoculeerd op een toetsplantenreeks waarop TMV en TRV konden worden onderscheiden. In geen van de toetsplanten werden de voor TRV-infectie typische symptomen waargenomen.

Dat TRV ook niet aanwezig was in de vorm van vrij TRV-RNA bleek uit het feit dat fenol-bentoniet extracten van systemisch geïnfecteerde delen van 'Samsun NN' evenmin als gezuiverd TMV-RNA lokale lesies veroorzaakten op 'Bataaf' bonen. Fenol-bentoniet extract uit planten met TRV gaf wel lokale lesies op dit bonenras (Tabel 5). Alle RNA-suspensies gaven op 'Samsun NN' systemische symptomen, die voor TRV-RNA overigens duidelijk verschilden van die van de overige inocula (Tabel 5, Fig. 8).

Hypothese 5: *Groeiomstandigheden van de planten.* Drie factoren zijn in beschouwing genomen, te weten grond, water en pesticiden.

Grond. 'Samsun NN'-planten werden opgekweekt in grond van verschillende her-

komsten. Een deel van deze proeven werd uitgevoerd in Wageningen met handelsgrond, een ander deel in Baarn met ter plaatse bereide grond. De planten die opgekweekt waren in grond uit Baarn hadden lichtere bladeren en stengels met veel langere internodiën dan de planten in de grond in Wageningen. In Baarn werd een serie 'Samsun NN' geïnoculeerd met een gezuiverde TMV-suspensie. Vier van de 25 planten gaven systemische necrose te zien in de kas, waar de temperatuur soms steeg van 24°C tot 26°C. Ter vergelijking werden gezonde 'Samsun NN'-planten in potten overgebracht van Baarn naar Wageningen, waar zij werden verdeeld over de klimaatkamers van 18°C en 22°C en twee kassen. Alle planten werden geïnoculeerd met een gezuiverde suspensie van TMV-Wageningen. Na acht dagen vertoonden slechts enkele planten in de kassen systemische symptomen. In de klimaatkamers waren meer planten systemisch geïnfecteerd, maar ook hier was het aantal veel geringer dan bij planten opgekweekt in Wageningse grond.

Water. Daar de klimaatkamers in Wageningen hun water uit andere bron betrekken dan de kassen, en op beide plaatsen 'Samsun NN' en *N. glutinosa*-planten geïnfecteerd raakten, was het niet nodig water van nog andere herkomst bij de proeven te betrekken.

Pesticiden. De reacties van een serie 'Samsun NN'-planten die bespoten waren met verschillende pesticiden zijn vergeleken met die van planten die nooit met pesticiden in aanraking waren geweest. In beide series kwamen evenveel systemisch-zieke planten voor.

Geen van de onderzochte factoren blijkt geheel verantwoordelijk te zijn voor optreden van de systemische reactie, al speelt de grond een belangrijke rol.

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