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Production of tobacco mosaic virus in and its infectivity from leaves of two Nicotiana species treated with 6-azauracil

JEANNE DIJKSTRA

Laboratorium voor Virologie der Landbouwhogeschool, Wageningen

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Abstract

A correlation between the amount of virus as determined by infectivity test and that determined by a serological-spectrophotometric method was established in leaf discs of *Nicotiana tabacum* 'White Burley' which had floated on a solution of 6-azauracil (AzU) before and after inoculation with tobacco mosaic virus. More virus, determined in the two different ways, was present in the AzU- than in the water-treated leaf discs at 24 and 48 h after inoculation. Thereafter, the amount of virus was less than that in the control. The inhibitory effect of the pyrimidine analogue on the amount of infectivity in sap from AzU-treated discs proved to be dependent on the dilution of sap used. The higher the dilution the more inhibition occurred.

In inoculated leaf discs of *Nicotiana glutinosa* the formation of local lesions in the AzU-treated series was strongly inhibited at 48 h after inoculation whereas at that time the amount of virus determined by the serological-spectrophotometric method was higher than that in the water-treated control ones. The latter mentioned stimulating effect disappeared later than 48 h after inoculation and gave way to inhibition.

The infectivity of tobacco mosaic virus from AzU-treated leaf discs of 'White Burley' tobacco was 23% of that from water-treated controls when undiluted clarified sap was used.

Introduction

An earlier paper (Dijkstra and van Rensen, 1968) showed a marked effect of 6-azauracil (AzU) on infection with tobacco mosaic virus (TMV) in *Nicotiana* species. The aim of the present study was to further analyse this effect.

The amount of virus formed in AzU- and water-treated leaf discs of *N. glutinosa* and *N. tabacum* "White Burley" was determined in two different ways, viz. by infectivity test and by a serological-spectrophotometric method (henceforth for the sake of brevity referred to as "serological method" only). In this way a distinction could be made between the infectivity on one hand and the amount of TMV-RNA associated with the virus protein on the other.

It is known that TMV formed in 2-thiouracil-treated tobacco leaves is much less infectious than that from leaves treated with water (Francki and Matthews, 1959; Francki, 1962). The same phenomenon has been observed in the case of TMV from tobacco leaves treated with 8-azaguanine (Matthews, 1954). In the present study the infectivity of TMV produced in AzU-treated leaf discs was compared with that in water-treated controls.

Materials and methods

Leaf discs, 18 mm in diameter, of *N. glutinosa* and *N. tabacum* 'White Burley' were floated with their lower surface on solutions of AzU at 4×10^{-4} M or on distilled water in the dark for 24 h. Thereafter, the discs were inoculated on their upper surface with 5 μg/ml of the purified local isolate 'Wageningen' of TMV. Excess inoculum was washed off under running tap water and the discs were floated again on fresh solutions of AzU and water, this time under continuous illumination from fluorescent tubes at a temperature of 18 °C ±1° for various periods, ranging from 24 to 144 h.

Epidermis material was stripped from the underside of *N. glutinosa* leaves which had floated on AzU or water for 24 h prior to inoculation with TMV. The strips were then maintained either on the AzU solution or on water under continuous illumination for 72 h.

The amount of infectivity in 'White Burley' leaf discs was determined by grinding 3×24 discs that had floated on the AzU solution as described above and by assaying the sap obtained at different dilutions on 3×6 right half-leaves of *N. glutinosa*. The left half-leaves were inoculated with a control suspension obtained by grinding 3×24 discs that had floated on water.

A serological method was used for estimation of virus in sap from leaf discs and epidermis. This was a modification of the method developed by Matthews (1954) for estimating the amount of TMV-RNA combined with virus protein in small samples of leaf sap. Twenty-four leaf discs or strips of epidermal tissue collected from two leaves at a time, to which was added 25 mg of Na_2HPO_4 were ground in 1 ml 0.1 M phosphate buffer, pH 7. The suspension obtained was heated to 55 °C for 10 min and the green precipitate removed by centrifugation at 3000 r.p.m. Then, 0.5 ml of the supernatant fluid was mixed with 0.5 ml of an excess (previously determined) of TMV antiserum. The mixture was kept at room temperature for 0.5 — 1 h and then overnight in the refrigerator. The precipitate formed was centrifuged down, washed with 2 ml of 0.14 M NaCl and extracted with 0.5 ml of 2.0 M $HClO_4$. After another night in the refrigerator, the mixutre was centrifuged, the precipitate washed with 2 ml of 1.0 M $HClO_4$, again centrifuged and the two supernatant fluids were pooled. This pool contained the hydrolysis products of TMV-RNA. The concentration of these products was then measured by determining the extinction at 260 mμ and estimating the concentration from a standard curve of purified yeast-RNA in 1.0 M perchloric acid.

Results

Effect of AzU on the amount of virus at different times after inoculation

In leaf discs of N. glutinosa. By using the above-mentioned serological method for determination of the amount of virus it could be demonstrated that more virus was present in the AzU- than in the water-treated leaf discs up to 48 h after inoculation (Fig. 1). Later, the stimulating effect of AzU disappeared and changed into an inhibition which remained almost constant afterwards.

On the other hand, the formation of local lesions that were found in the AzU-treated leaf discs was greatly inhibited at 48 h after inoculation but this inhibition

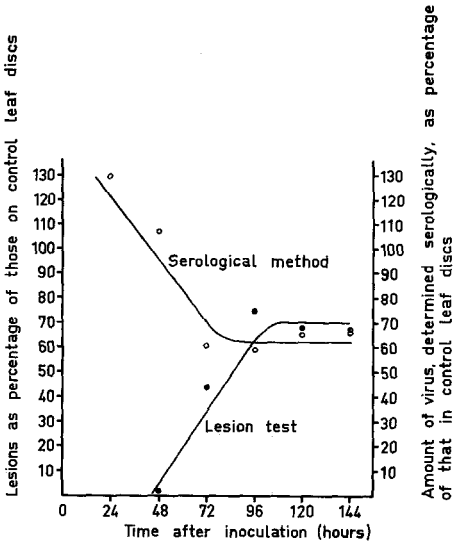

Fig. 1. The effect of 6-azauracil (AzU) on the number of local lesions and on the amount of virus determined by a serological method in leaf discs of *Nicotiana glutinosa* inoculated with TMV.

The concentrations of the AzU solution and the TMV suspensions were 4×10^{-4} M and 5 μg/ml, respectively. The number of local lesions and the amount of virus in AzU-treated leaf discs are given as average percentage of those in water-treated controls.

Fig. 1. Het effect van 6-azauracil (AzU) op het aantal lokale lesies en de langs serologische weg bepaalde hoeveelheid virus in bladschijfjes van Nicotiana glutinosa, die waren geïnoculeerd met TMV.

decreased and reached a more or less constant level at 96 h after inoculation (Fig. 1).

In leaf discs of N. tabacum 'White Burley'. A greater amount of virus as estimated by the serological method and by infectivity test was observed in the AzU-treated leaf discs up to 48 h after inoculation (Fig. 2). Later, this stimulation gave way to inhibition.

The relative infectivity of freshly extracted sap obtained from ground leaf discs was determined at different dilutions viz. undiluted, 1:2, 1:10, 1:100, 1:500. From Table 1

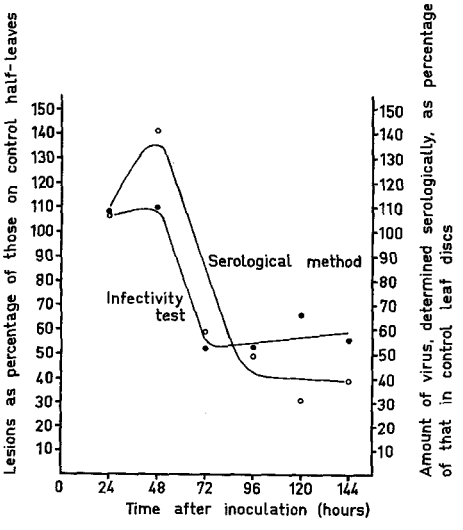

Fig. 2. The effect of 6-azauracil (AzU) on the amount of virus determined by infectivity test and by a serological method in leaf discs of *Nicotiana tabacum* 'White Burley' inoculated with TMV.

The concentrations of the AzU solution and the TMV suspensions were 4×10^{-4} M and 5 μg/ml, respectively. Bio-assay was carried out by grinding leaf discs of tobacco that had floated on the AzU solution and assaying the sap obtained at dilutions 1:2 (in the 24 and 48 h series) and 1:10 (in the 72, 96, 120 and 144 h series) onto right half-leaves of *N. glutinosa*. The left half-leaves were inoculated with control suspensions obtained by grinding leaf discs of tobacco that had floated on water. The number of local lesions on the assay halves is expressed as average percentage of that on the control halves.

Fig. 2. Het effect van 6-azauracil (AzU) op de hoeveelheid virus, bepaald met behulp van een infectietoets en van een serologische methode, in bladschijfjes van Nicotiana tabacum 'White Burley', die waren geïnoculeerd met TMV.

Table 1. Effect of dilution of sap from 6-azauracil (AzU)-treated leaf discs of *Nicotiana tabacum* 'White Burley' inoculated with TMV, on the number of local lesions on assay half-leaves of *Nicotiana glutinosa*.

Time after inoculation at which leaf discs were assayed (hours)	Relative infectivity[1] of sap from AzU- and water-treated leaf discs, assayed at different dilutions				
	undiluted	1:2	1:10	1:100	1:500
24	147	107	–	–	–
48	128	110	–	–	–
72	–	–	52	41	–
96	–	–	53	37	30
120	–	–	66	42	26
144	–	–	56	40	28

[1] The number of local lesions on assay half-leaves of *N. glutinosa* inoculated with sap from AzU-treated leaf discs of *N. tabacum* expressed as percentage of that on control half-leaves of *N. glutinosa* inoculated with sap from water-treated leaf discs of *N. tabacum* (mean of 3 experiments in the 24, 48, 72 and 144 h series; mean of 4 experiments in the 96 and 120 h series).

Tabel 1. Effect van verdunning van sap uit met TMV geïnoculeerde bladschijfjes van Nicotiana tabacum 'White Burley', die op 6-azauracil (AzU) hadden gedreven, op het aantal lokale lesies op toetsbladhelften van Nicotiana glutinosa.

it can be seen that in the 24 and 48 h series the stimulating effect of AzU on the number of local lesions on the assay half-leaves decreased, and in the 72, 96, 120 and 144 h series the inhibiting effect of AzU increased at increasing sap dilutions.

In isolated epidermal tissue of N. glutinosa leaves. Isolated epidermal tissue in which the amount of virus was determined with the serological method at 72 h after inoculation contained more TMV in the AzU-treated series than in the water-treated control one. However, as the absolute amount of virus recovered from this tissue was very low, an exact quantitative comparison was not possible. In two out of the four experiments carried out the concentration of the virus was too low to be determined spectrophotometrically.

Infectivity of TMV cultured in the presence of AzU

A comparison between the infectivity of TMV from AzU-treated leaf discs of 'White Burley' and that from water-treated discs was made 72 h after inoculation.

Out of 72 leaf discs, AzU- or water-treated, 24 were ground and the freshly extracted sap immediately assayed onto six *N. glutinosa* half-leaves at three dilutions (undiluted, 1:2, 1:4). As the concentration of the virus in this sap was not known at the time of assaying, a correction was made by comparing only the results obtained with those sap dilutions in the two series that had comparable virus concentrations, as determined serologically in the following.

The remaining 48 leaf discs were ground and the sap obtained was clarified by heating and low speed centrifugation as described under *Materials and methods*. The clarified sap was divided into two equal portions which were stored at −10 °C for 2 days. One of these portions was then thawed and an estimation of its virus content was made by the serological method. As soon as the concentration of the TMV-RNA

Table 2. The effect of 6-azauracil (AzU) on the relative infectivity of TMV.

Assay carried out on	Relative infectivity[1] of sap from AzU- and water-treated leaf discs, assayed at different dilutions		
	undiluted	1:2	1:4
Freshly extracted sap	45	34	–
Clarified sap	23	23	25

[1] The number of local lesions on assay half-leaves of *Nicotiana glutinosa* inoculated with sap from AzU-treated leaf discs of *N. tabacum* expressed as percentage of that on control half-leaves of *N. glutinosa* inoculated with sap from water-treated leaf discs of *N. tabacum* (mean of 3 experiments when freshly extracted sap was used; mean of 10 experiments in the case of clarified sap). The inocula prepared from control and AzU-treated leaf discs were equalized with respect to their TMV-RNA concentrations.

Tabel 2. Het effect van 6-azauracil (AzU) op de relatieve infectiositeit van TMV.

was determined in the two series, the other frozen portions were thawed and the TMV-RNA concentrations in them equalized. Thereafter, the suspensions with equalized concentrations were diluted and assayed for infectivity onto six *N. glutinosa* half-leaves.

It can be seen from Table 2 that in freshly extracted and clarified sap the infectivity of virus from the AzU-treated discs was much lower than that from the water-treated controls. The infectivity of virus in clarified sap was not affected by dilution.

Effects of sap from AzU-treated 'White Burley' leaf discs on lesion production by TMV in N. glutinosa

In order to rule out the possibility that the reduced infectivity of TMV, cultured in the presence of the analogue was due to the presence of an AzU-induced inhibitor in sap

Table 3. Effect of sap from healthy leaf discs of *Nicotiana tabacum 'White Burley'* treated with 6-azauracil (AzU) on lesion production by TMV in *Nicotiana glutinosa*.

No. of experiment	Number of lesions per half-leaf[1]		% lesion numbers AzU: control
	Control[2]	AzU[3]	
	462	452	98
1	425	502	118
	610	628	103
	587	560	95
2	328	419	128
	669	672	100

[1] Each experiment was carried out in triplicate. For each assay, 12 half-leaves were used.

[2, 3] Sap from leaf discs floated on water (control) and AzU, respectively, was mixed 1:1 with a standard purified TMV suspension of 5 µg/ml.

Tabel 3. Effect van sap uit gezonde bladschijfjes van Nicotiana tabacum 'White Burley', die waren behandeld met 6-azauracil (AzU), op de door TMV te produceren lokale lesies op Nicotiana glutinosa.

from AzU-treated leaf discs, the following experiment was done. Leaf discs, floated on AzU or water, were 'inoculated' with distilled water, ground 72 h after 'inoculation and the sap mixed 1:1 with a standard purified TMV suspension of 5 μg/ml. The mixture was assayed onto 12 half-leaves of *N. glutinosa*. Table 3 shows that sap from healthy 'White Burley' leaf discs treated with AzU has no inhibitory effects on the production of local lesions by TMV in *N. glutinosa*.

Discussion

In contrast to what has been reported earlier (Dijkstra and van Rensen, 1968), in most of the present experiments no reduction in the amount of infectivity was observed in AzU-treated leaf discs of 'White Burley' tobacco up to 48 h after inoculation. On the contrary, at 48 h after inoculation a marked stimulation of both the amount of virus determined serologically and the amount of infectivity established by infectivity test occurred in the AzU-treated discs. These differences were probably due to the differences in age of the material used in the two investigations. In the earlier work, big leaves (about 20 cm in length) from old plants were used whereas in the present experiments leaves not longer than 10 cm from very young tobacco plants.

The amount of virus determined serologically in AzU-treated leaf discs of *N. glutinosa* also revealed a marked stimulation at 24 h and a less conspicuous one at 48 h after inoculation.

Isolated epidermal tissue of *N. glutinosa* treated with either AzU or water did not show the presence of virus, determined serologically, earlier than 72 h after inoculation. This is understandable, since stripping of the epidermis affects the physiological processes in the cells delaying virus synthesis. However, in spite of the very small amounts of virus recovered from both control and test samples by which it was impossible to determine absolute virus contents, at 72 h after inoculation a greater amount of virus was observed in AzU-treated tissue than in the control. Earlier, it has been shown that the amount of infectivity in the AzU-treated epidermal tissue was also higher than in the control one (Dijkstra and van Rensen, 1968).

On the basis of the above-mentioned results the hypothesis may be launched that during the first 48 h after inoculation synthesis of TMV in AzU-treated leaves takes place mainly in the epidermal cells where AzU does not seem to act as an inhibitor but acts rather as a stimulator of infection. The dominant role of the epidermis in the synthesis of TMV might be due to a delayed transport of virus from the epidermal cells to the underlayers of AzU-treated leaves. Such a hampered transport might also be the cause of the reduced speed of lesion development on AzU-treated leaf discs of *N. glutinosa* at 48 h after inoculation, as has been suggested earlier (Dijkstra and van Rensen, 1968). Experiments with 8-azaguanine have shown that systemic spread of TMV was inhibited much more than could be accounted for by inhibition of replication sites (Lindner et al., 1960).

On the other hand, experiments in which the infectivity of TMV from AzU-treated leaf discs of 'White Burley' tobacco was compared with that from water-treated control discs at equalized sap concentrations revealed that the infectivity in the former case was much less. An explanation could be that AzU is incorporated into TMV-RNA which thus becomes incapable of replication.

From the above, it must be concluded that accumulation of virus in AzU-treated epidermal cells makes up for the reduced infectivity of TMV.

An explanation for the fact that the relative infectivity of freshly extracted sap from leaf discs floated on the analogue decreased at increasing sap dilutions might be that the non-infectious virus particles are aggregated in the undiluted suspension. At increasing dilution these particles are disaggregated and are able to occupy the susceptible sites on the leaf at the expense of the infectious particles.

The reduced virus production in leaf discs of 'White Burley' from 72 h onward might be attributed to a hampered transport from cell to cell. In addition, the RNA synthesis might also be inhibited in the presence of AzU, as demonstrated by Handschumacher and Pasternak (1958) who studied the carcinostatic and bacteriostatic activity of AzU and have advanced the hypothesis that AzU is converted into riboside-containing metabolites. These metabolites inhibit the enzyme orotidylic decarboxylase which converts orotidine-5'-phosphate into uridine-5'-phosphate so that RNA synthesis can not take place. Recently, a support for this hypothesis has been given by Dekker (1968) who could demonstrate that the resistance of some mutant strains of *Cladosporium cucumerinum* was due to the inability of the latter to convert AzU into one of the RNA synthesis inhibiting compounds.

Regarding the absence of inhibition of virus synthesis during the first 24 and 48 h after inoculation (synthesis presumably being mainly in the inoculated epidermis) and the presence of inhibition of virus synthesis later than 48 h (synthesis presumably being mainly in the underlying tissues) in AzU-treated leaves it is thinkable that this is caused by inability of epidermal cells to convert AzU into its riboside metabolites so that blocking of RNA synthesis does not take place.

Acknowledgment

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Samenvatting

Produktie van tabaksmozaïekvirus en zijn infectiositeit in met 6-azauracil behandelde bladeren van twee Nicotiana-soorten

In een vroegere publikatie (Dijkstra en van Rensen, 1968) was aangetoond, dat 6-azauracil (AzU) een duidelijke invloed heeft op de infectie van *Nicotiana*-soorten met tabaksmozaïekvirus (TMV). Het doel van het huidige onderzoek was om dit effect nader te analyseren.

In bladschijfjes van *N. glutinosa*, die met TMV waren geïnoculeerd en gedreven hadden op AzU of water, was 48 uur na inoculatie de vorming van lokale lesies in de met AzU behandelde toetsserie sterk geremd, terwijl daarentegen op dat tijdstip de hoeveelheid virus bepaald langs serologische-spectrofotometrische (kortheidshalve in het vervolg aangeduid als "serologische") weg hoger was dan die in de met water behandelde controleserie (Fig. 1).

In bladschijfjes van *N. tabacum* 'White Burley', die op AzU of water hadden gedreven, werd een correlatie gevonden tussen de hoeveelheid virus bepaald langs biologische weg (infectietoets) en die welke met de serologische methode was vastgesteld.

Met beide methodes kon op de tijdstippen 24 en 48 uur na inoculatie meer virus worden aangetoond in de AzU serie dan in de controlereeks (Fig. 2). Na laatstgenoemd tijdstip daalde de hoeveelheid virus in de toetsserie tot beneden het niveau van de controleserie. De vermindering van de infectiositeit van sap afkomstig van bladschijfjes die met AzU waren behandeld bleek afhankelijk te zijn van de verdunning van het sap: hoe groter de verdunning des te sterker de remming (Tabel 1).

De infectiositeit van TMV uit gedeeltelijk gezuiverd sap van bladschijfjes van 'White Burley', die met AzU waren behandeld, bleek slechts 23% te bedragen van die van de met water behandelde controles (Tabel 2).

In sap van de met AzU behandelde bladschijfjes van 'White Burley' bleek geen door de pyrimidine-analoog geïnduceerde remstof aanwezig te zijn (Tabel 3).

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