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Effect of 6-azauracil on infection with tobacco mosaic virus[1]

Jeanne Dijkstra and J. J. S. van Rensen[2]

Laboratory of Virology, Agricultural University, Wageningen

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Abstract

6-Azauracil caused a marked reduction in the number and size of local lesions on excised *Nicotiana glutinosa* leaves or leaf discs inoculated with tobacco mosaic virus or its nucleic acid. The amount of infectivity recovered from tobacco (*N. tabacum* 'White Burley') leaf discs floated on the pyrimidine analogue solution and subsequently ground and assayed 24, 48, 72, and 96 h after inoculation with intact virus was reduced in the 24, 48, and 72 h series but reached the same level as that of the water-treated control discs in the 96 h series.
By contrast, the amount of infectivity in stripped epidermal tissue of *N. glutinosa* leaves inoculated with nucleic acid was not reduced in strips floated on the analogue for 24, 50, and 70 h as compared with water-floated controls. The analogue had no effect on infectivity of the virus in vitro and did not act as an inhibitor of infection.

Introduction

Substances known to interfere with the infection and multiplication processes of viruses are of both practical and theoretical interest. In this connection, inhibitors structurally closely related to virus nucleic acid constituents are attracting special attention. Consequently, many investigations have already been carried out on the effects of purine and pyrimidine analogues such as, for example, 2-thiouracil (Ralph et al., 1963) and 8-azaguanine (Matthews and Smith, 1955).
In this paper results are reported of experiments on the effect of 6-azauracil (AzU) on infection with tobacco mosaic virus (TMV) or its nucleic acid (TMV-NA). This pyrimidine analogue has bacteriostatic (Škoda and Šorm, 1958) and carcinostatic (Handschumacher and Pasternak, 1958) effects. Moreover, preliminary investigations of Dekker (1962) and Dekker and Oort (1964) pointed to a marked inhibitory effect of AzU on infection with TMV in leaf discs of *Nicotiana glutinosa*.

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[2] Present address: Laboratory of Plant Physiological Research, Agricultural University, Wageningen.

Materials and methods

The effect of AzU was studied with excised leaves of *N. glutinosa* and leaf discs, 18 mm in diameter, of *N. glutinosa* and *N. tabacum* 'White Burley' which were floated either with their upper surface or with their lower surface on solutions of AzU at 4×10^{-4}M. A purified local isolate of TMV was prepared from leaves of 'White Burley' plants by a standard method using acid precipitation of the virus from the clarified sap followed by differential centrifugation. The suspension obtained was stored at a temperature of about 4 °C.

Floating of leaves or leaf discs on water or AzU solution took place either before and after inoculation or only after inoculation with TMV. Further details are given in the legends to Fig. 1, 2 and 3.

In some experiments isolated epidermal tissue floated on the analogue was assayed for infectivity. According to the following procedure leaves of *N. glutinosa* were floated with their lower surface on AzU for 24 h. They were then inoculated with TMV-NA on their lower surface and immediately deprived of the lower epidermis according to the method described by Dijkstra (1964). The epidermis strips were then maintained either on the analogue solution or on water for various periods (0, 24, 50, and 70 h). Thereafter, epidermis strips from two leaves at a time were collected, ground without additional liquid and assayed on six excised *N. glutinosa* leaves. The latter were kept on moist filter paper under continuous fluorescent light.

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

Results and discussion

The preliminary findings of Dekker (1962) and Dekker and Oort (1964) could be confirmed by us, using a TMV inoculum of 5 or 10 μg/ml but we found the inhibition to be dependent on the time at which the lesion counts were made: at 48 h after inoculation (the time used by the above authors for lesion counting) inhibition was complete but it decreased if the experiment was extended (Fig. 1). Further the effect of the analogue was most pronounced if the leaf discs were rubbed with water and carborundum on the lower surface, and floated on AzU on the same side for 24 h prior to inoculation on the lower surface. Inoculation preceded by a floating period of 24 h and followed by another one of 144 h always resulted in a smaller number of lesions than a similar treatment without a pre-floating period.

Similar results were obtained if TMV-NA instead of intact TMV served as inoculum. When intact leaves instead of leaf discs were used, the same trend could be observed but the differences between the various treatments were less distinct (Fig. 2).

Another striking phenomenon was that lesions developing on AzU-treated leaf tissue were 2/3 or 1/2 as wide as those on water-treated controls, especially during the first three days after inoculation.

In order to compare the effect of the analogue in a local lesion test plant with that in a systemically infected one, leaf discs were taken from 'White Burley' plants. These were at first floated on the analogue solution with the lower surface for 24 h, then inoculated on the upper surface and floated again for various periods. Subsequently, a number of leaf discs were ground and the sap assayed on *N. glutinosa* leaf-halves. Fig. 3 shows

Fig. 1. The effect of 6-azauracil (AzU) on the number of local lesions on leaf discs of *Nicotiana glutinosa* inoculated with TMV suspensions.
The concentrations of the AzU solution and the TMV suspensions were 4×10^{-4}M and 5 or 10 μg/ml, respectively.
The number of local lesions on 4×6 AzU-treated leaf discs is given as percentage of that produced on 4×6 water-treated controls.

1 Leaf discs inoculated on their lower surface and floated with their lower surface on AzU and water, respectively (mean of 10 experiments)
2 Leaf discs inoculated on their upper surface and floated with their lower surface on AzU and water, respectively (mean of 15 experiments)
3 Leaf discs floated with their lower surface on AzU and water, respectively, for 24 h prior to inoculation on their upper surface (mean of 23 experiments)
4 Leaf discs floated with their lower surface on AzU and water, respectively, for 24 h prior to inoculation on their lower surface (mean of 11 experiments)
5 Leaf discs rubbed with water and carborundum on their lower surface and floated with their lower surface on AzU and water, respectively, for 24 h prior to inoculation on their lower surface (mean of 6 experiments)

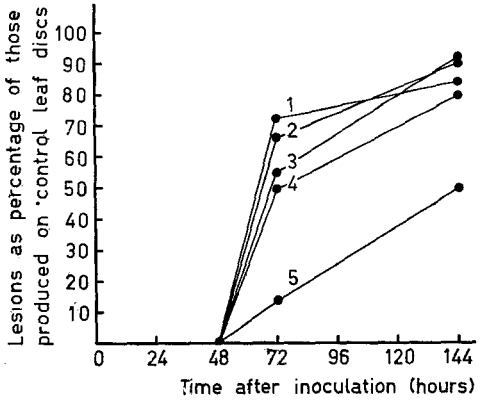

Fig. 1. Het effect van 6-azauracil (AzU) op het aantal lokale lesies op bladschijfjes van Nicotiana glutinosa geïnoculeerd met TMV-suspensies.
De concentraties van de AzU oplossing en de TMV-suspensies waren resp. 4×10^{-4}M en 5 of 10 μg/ml. Het aantal lokale lesies op 4×6 met AzU behandelde bladschijfjes is weergegeven als percentage van dat op 4×6 met water behandelde controles.

1 Bladschijfjes geïnoculeerd op hun onderkant en met hun onderkant te drijven gelegd op resp. AzU en water (gemiddelde van 10 proeven)
2 Bladschijfjes geïnoculeerd op hun bovenkant en met hun onderkant te drijven gelegd op resp. AzU en water (gemiddelde van 15 proeven)
3 Bladschijfjes gedurende 24 uur met hun onderkant te drijven gelegd op resp. AzU en water vóór inoculatie op hun bovenkant (gemiddelde van 23 proeven)
4 Bladschijfjes gedurende 24 uur met hun onderkant te drijven gelegd op resp. AzU en water voor inoculatie op hun onderkant (gemiddelde van 11 proeven)
5 Bladschijfjes op hun onderkant ingewreven met water en carborundum en gedurende 24 uur met hun onderkant te drijven gelegd op resp. AzU en water vóór inoculatie op hun onderkant (gemiddelde van 6 proeven)

Fig. 2. The effect of 6-azauracil (AzU) on the number of local lesions on leaves of *Nicotiana glutinosa* inoculated with TMV suspensions.
The concentrations of the AzU solution and the TMV suspensions were 4×10^{-4}M and 5 or 10 μg/ml, respectively.
The number of local lesions on 4×6 AzU-treated leaves is given as percentage of that produced on water-treated controls.
For explanation of the curves 1–5 see legend to Fig. 1 in which "leaf discs" need to be replaced by "leaves" and the number of experiments by 4, 7, 4, 10 and 10, respectively.
6 Leaves rubbed with water and carborundum on their lower surface and floated with their lower surface on AzU and water, respectively, for 24 h prior to inoculation on their upper surface (mean of 5 experiments)

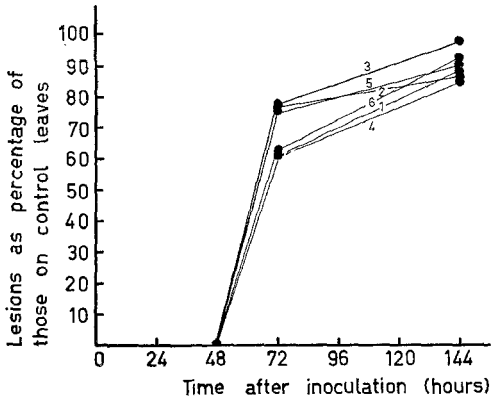

Fig. 2. Het effect van 6-azauracil (AzU) op het aantal lokale lesies op bladeren van Nicotiana glutinosa geïnoculeerd met TMV-suspensies.
De concentraties van de AzU oplossing en de TMV-suspensies waren resp. 4×10^{-4} M en 5 of 10 μg/ml.
Het aantal lokale lesies op 4×6 met AzU behandelde bladeren is weergegeven als percentage ten opzichte van de met water behandelde controles.
Voor verklaring van de curven 1–5 zie onderschrift bij Fig. 1, waarin "bladschijfjes" vervangen dienen te worden door "bladeren" en het aantal proeven door resp. 4, 7, 4, 10 en 10.
6 Bladeren op hun onderkant ingewreven met water en carborundum en gedurende 24 uur met hun onderkant te drijven gelegd op resp. AzU en water vóór inoculatie op hun bovenkant (gemiddelde van 5 proeven)

that the infectivity in the AzU-treated leaf discs was reduced in the 24, 48, and 72 h series but reached the same level as that of the water-treated controls in the 96 h series. The results with isolated epidermal tissue are given in Fig. 4 and 5. No reduction of the amount of infectivity in epidermis strips floated on the analogue could be observed; at 70 h the amount of infectivity in the analogue-treated epidermal tissue was even higher (at 5% significance level) than in the water-treated control (Fig. 4). however, the lower surfaces of the test leaves were rubbed with water and carborundum prior to floating on the solution and inoculation with TMV-NA, less infectivity could be established in the analogue-treated series than in the control (Fig. 5). This phenomenon might be explained by assuming that in the latter case the analogue had affected the infectible sites. This explanation might also hold good for the leaf discs of *N. glutinosa*, to which a pretreatment with water and carborundum was given prior to floating on the analogue and inoculation, showing much less local lesions at 72 and 144 h after inoculation than those without such a pretreatment (Fig. 1).

Fig. 3. The effect of 6-azauracil (AzU) on the amount of infectivity recovered from leaf discs of *Nicotiana tabacum* 'White Burley' floated on the above-mentioned solution and inoculated with TMV suspensions.
Analyses were carried out at various times after inoculation. The concentrations of the AzU solution and the TMV suspensions were 4×10^{-4} M and 5 μg/ml, respectively.
The amount of infectivity was established by grinding 4×6 leaf discs of tobacco and assaying the sap obtained on 4×6 right leaf-halves of *N. glutinosa*. The left leaf-halves were inoculated with a standard TMV suspension of 5 μg/ml and served as control.
The number of local lesions on the assay halves is expressed as average percentage of that on the control halves.
The ratio of the average percentage obtained with leaf discs floated on AzU to the average percentage obtained with leaf discs floated on water is multiplied by 100 and plotted against the time after inoculation at which samples were analysed. Each point presents the mean of 4 experiments.

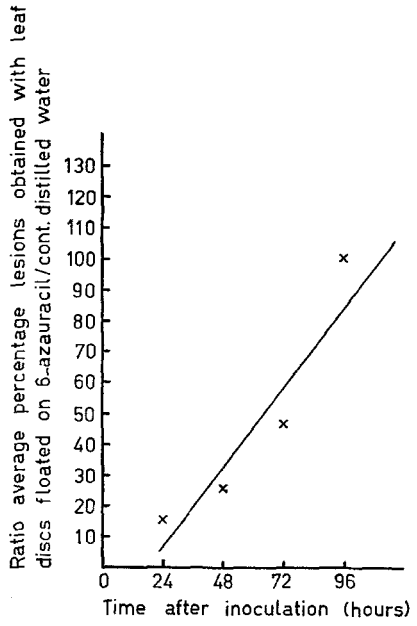

Fig. 3. Het effect van 6-azauracil (AzU) op de hoeveelheid infectiositeit van bladschijfjes van Nicotiana tabacum 'White Burley' na drijven op een oplossing van bovengenoemde stof en inoculatie met TMV-suspensies.
Analyses werden op verschillende tijdstippen na inoculatie uitgevoerd. De concentraties van de AzU oplossing en de TMV-suspensies waren resp. 4×10^{-4}M en 5 μg/ml.
De hoeveelheid infectiositeit werd bepaald door 4 × 6 bladschijfjes van 'White Burley' fijn te wrijven en het sap te toetsen op 4 × 6 rechter bladhelften van N. glutinosa. De linker bladhelften werden geïnoculeerd met een standaard-TMV-suspensie van 5 μg/ml en dienden als controle. Het aantal lokale lesies op de toetshelften is weergegeven als gemiddeld percentage ten opzichte van dat op de controle-helften. De verhouding van het gemiddelde percentage, verkregen met bladschijfjes die op AzU hebben gedreven, tot het gemiddelde percentage, verkregen met bladschijfjes die op water hebben gedreven, is vermenigvuldigd met 100 en afgezet tegen het tijdstip na inoculatie waarop de monsters werden geanalyseerd. Elk punt geeft het gemiddelde weer van 4 proeven.

Fig. 4. The effect of 6-azauracil (AzU) on the amount of infectivity recovered from epidermal tissue removed from *Nicotiana glutionosa* leaves. The latter were floated on the test solution (solid line) or distilled water (dashed line) with their lower surface for 24 h prior to inoculation on the same surface with TMV-nucleic acid.
The concentration of the AzU solution was 4×10^{-4}M. Infectivity was established by collecting and grinding epidermis strips from 6 test leaves at a time and by assaying this sample on 6 right leaf-halves of *N. glutinosa*. The left halves were inoculated with a standard TMV suspension of 2μg/ml. Each point presents the mean of 5 experiments.

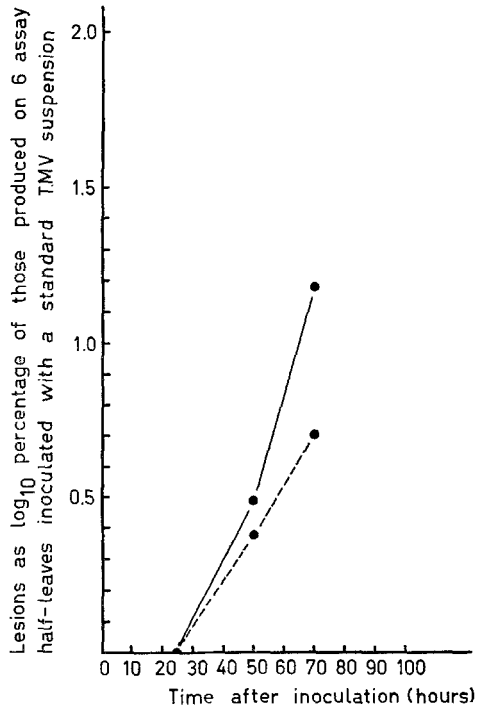

Fig. 4. Het effect van 6-azauracil (AzU) op de infectiositeit van met TMV geïnoculeerd epidermisweefsel afkomstig van bladeren van Nicotiana glutinosa. De bladeren hadden gedurende 24 uur met hun onderkant gedreven op de toetsoplossing (getrokken lijn) of gedestilleerd water (onderbroken lijn) vóór inoculatie op dezelfde kant met nucleïnezuur van TMV.
De concentratie van de AzU oplossing was 4 × 10^{4} M. De hoeveelheid infectiositeit werd bepaald door stukjes epidermis afkomstig van 6 proefbladeren gezamenlijk te toetsen op 6 rechter bladhelften van N. glutinosa. De linker helften werden geïnoculeerd met een standaard-TMV-suspensie van 2 μg/ml. Elk punt geeft het gemiddelde weer van 5 proeven.

Experiments in which a solution of AzU (8×10^{-4}M) was mixed 1:1 with a TMV suspension (5 or 10 μg/ml) and the mixture assayed on excised *N. glutinosa* leaves proved that the analogue had no effect on infectivity of the virus in vitro and did not act as an inhibitor of infection by rendering the infectible sites unfit for receiving the virus particles, when applied simultaneously with the virus suspension.
The above-mentioned results from experiments with *N. glutinosa* and tobacco leaves or leaf discs do not point unequivocally to an inhibitory effect of the analogue on the multiplication of the virus for it is possible that the transport from cell to cell slows down so that less cells become infected during some time after inoculation. Conse-

Fig. 5. The effect of 6-azauracil (AzU) on the amount of infectivity recovered from epidermal tissue removed from *Nicotiana glutinosa* leaves. The latter were rubbed with water and carborundum, and floated with their lower surface on the test solution (solid line) or distilled water (dashed line) for 24 h prior to inoculation on the same surface with TMV-nucleic acid.
The concentration of the AzU solution was 4×10^{-4}M. The amount of infectivity was established by collecting and grinding epidermis strips from 6 test leaves at a time and by assaying this sample on 6 excised *N. glutinosa* leaves. Each point presents the mean of 5 experiments.

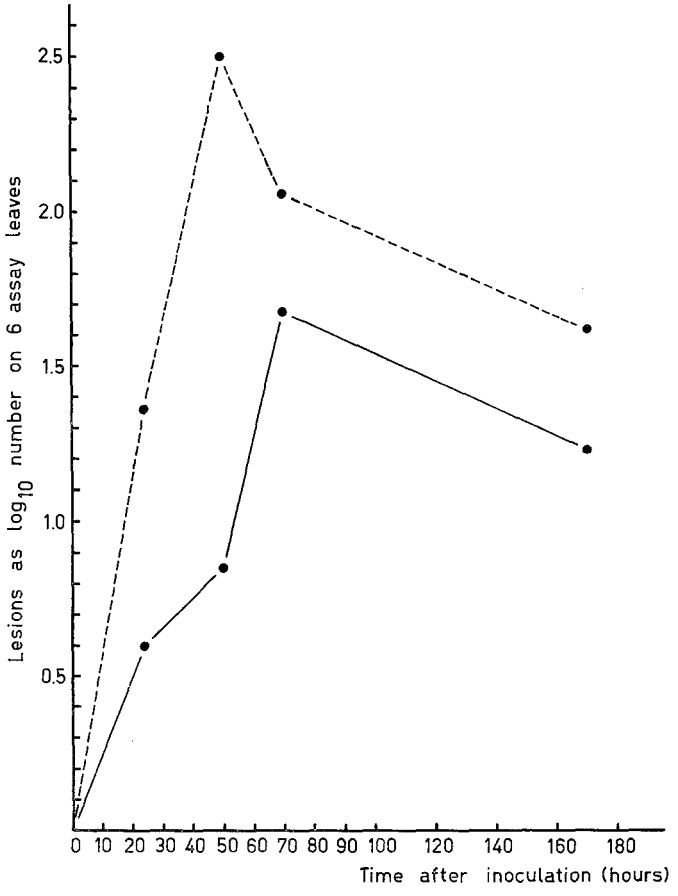

Fig. 5. Het effect van 6-azauracil (AzU) op de infectiositeit van met TMV geïnoculeerd epidermisweefsel afkomstig van Nicotiana glutinosa. De bladeren werden ingewreven met water en carborundum en gedurende 24 uur met hun onderkant te drijven gelegd op de toetsoplossing (getrokken lijn) of gedestilleerd water (onderbroken lijn) vóór inoculatie op dezelfde kant met nucleïnezuur van TMV.
De concentratie van de AzU oplossing was 4×10^{-4}M. De hoeveelheid infectiositeit werd bepaald door stukjes epidermis afkomstig van 6 proefbladeren te verzamelen, fijn te wrijven en het aldus verkregen sap te toetsen op 6 afgeknipte bladeren van N. glutinosa. Elk punt geeft het gemiddelde weer van 5 proeven.

quently, the total amount of virus produced is less. Such a reduced transport is also suggested by results obtained with isolated epidermal tissue floated on the analogue and by the reduced speed of development of lesions (Fig. 1) and size of lesions.

Samenvatting

Effect van 6-azauracil op infectie met tabaksmozaïekvirus

Uit proeven van Dekker (1962) en Dekker en Oort (1964) was gebleken, dat het aantal lokale lesies op bladschijfjes van *N. glutinosa* na inoculatie met tabaksmozaïekvirus (TMV) sterk verminderd was, indien de schijfjes vóór inoculatie hadden gedreven op een oplossing van 6-azauracil (AzU).
Hun resultaten konden door ons worden bevestigd, doch de afname van het aantal lesies bleek afhankelijk te zijn van het tijdstip waarop ze werden geteld. Werden de tellingen verricht meer dan 48 uur na inoculatie (het tijdstip waarop bovengenoemde auteurs hadden geteld) dan bleek nl. het verschil tussen het aantal lesies op de met AzU en water behandelde bladeren of bladschijfjes hoe langer hoe kleiner te worden (Fig. 1 en 2).
De diameter van de lokale lesies die op de met AzU behandelde bladeren of bladschijfjes verschenen, was gewoonlijk slechts 2/3 tot 1/2 maal zo groot als die van de met water behandelde controles.
Proeven met tabak (*N. tabacum* 'White Burley') wezen uit, dat de infectiositeit van bladschijfjes, die gedurende 24 uur met hun onderkanten hadden gedreven op AzU en vervolgens waren fijngemaakt en getoetst op bladhelften van *N. glutinosa*, 24, 48, 72 en 96 uur na inoculatie met TMV, minder was in de 24, 48 en 72 uur groep doch in de 96 uur groep het zelfde niveau bereikte als die van de met water behandelde controle schijfjes (Fig. 3).
Het effect van de analoog werd ook bestudeerd aan geïsoleerd epidermisweefsel van bladeren van *N. glutinosa*. Daartoe werden de bladeren met hun onderkant te drijven gelegd op resp. AzU oplossing en water, en vervolgens op dezelfde kant geïnoculeerd met TMV of het nucleïnezuur ervan (TMV-NA). Onmiddellijk na inoculatie werd van zes bladeren de onderepidermis verwijderd en te drijven gelegd op resp. AzU en water. Op verschillende tijdstippen na inoculatie werden de stukjes epidermis van telkens twee bladeren verzameld, fijngewreven en het aldus verkregen sap getoetst op zes afgeplukte bladeren van *N. glutinosa*. Uit deze proeven bleek dat de infectiositeit in epidermisweefsel dat gedreven had op AzU niet minder was dan die in de controle-stukjes epidermis, die op water hadden gedreven (Fig. 4). Dit laatste was echter wel het geval als de onderkanten van de bladeren van *N. glutinosa* vóór het drijven op de respectievelijke oplossingen en vóór inoculatie met TMV-NA waren ingewreven met water en carborundum (Fig. 5). Dit zou mogelijkerwijs het gevolg kunnen zijn van een aantasting van 'infectible sites' door de analoog.
Uit proeven, waarbij een AzU oplossing van 8×10^{-4}M in gelijke verhoudingen werd gemengd met een TMV-suspensie van 5 tot 10 µg/ml en het mengsel werd geïnoculeerd op afgeknipte bladeren van *N. glutinosa*, bleek, dat de analoog geen invloed had op de infectiositeit van het virus in vitro en evenmin fungeerde als "inhibitor of infection".

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