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A DEVICE FOR THE RAPID REMOVAL OF TANNINS FROM VIRUS INFECTED PLANT TISSUES BEFORE EXTRACTION OF INOCULUM[1)2)]

Met een samenvatting: Een methode voor de snelle verwijdering van looistoffen uit viruszieke plantedelen

BY

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The difficulty of extraction of certain plant viruses in infectious form is believed to be due to the denaturing effect of tannins liberated in the course of juice extraction. During the grinding or pressing of the tissues the tannins, localized in vacuoles of living cells, become mixed with the elements of cytoplasmic origin and inactivate the viruses.

CORNUET (1) has suggested that this difficulty may be avoided by effecting a non-denaturing dehydration of the material through freeze-drying followed by washing with ethyl alcohol or chloroform. In either case it is essential that the solvent be completely anhydrous, otherwise it will itself inactivate the virus or bring it into contact with the denaturing tannins.

Ordinary methods of washing and centrifuging have two inherent disadvantages: too much time (4 to 8 hours) is required when 7–10 washings are needed, and there is always the danger of atmospheric moisture being taken up by the absolute alcohol during the extraction process. In an effort to speed the extraction and reduce the danger of moisture getting into the solvent the Soxhlet extraction apparatus was tested. However, the temperature in the cellulose extraction thimble was too high (55–60 °C with chloroform; 70–75 °C with alcohol) and would quickly have inactivated most plant viruses. Modifications of the apparatus, on the other hand, removed the difficulties.

A water jacket (Fig. 1, 3) was placed around the chamber in which the cellulose thimble containing plant materials was to be placed, making it possible to keep the chamber at any desired temperature. To reduce the chances of absorbing moisture from the air a longer condensor (Fig. 1, 2) was built and a $CaCl_2$ trap (Fig. 1, 1) was added. Also, a test cock (Fig. 1, 4) was added to the siphon tube below the water jacket so that samples could be taken for testing with $FeCl_3$ without opening the apparatus or stopping the extraction. With this equipment, which was made at the Laboratory of Organic Chemistry, Landbouwhogeschool, from Pyrex glass, a rapid, repeated washing of the plant material was possible.

To determine whether such a device could be used in plant virus research,

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leaves of White Burley tobacco, infected with tobacco mosaic virus, and old leaves of commercial strawberry which were high in tannins, were freeze-dried, ground to a powder, and mixed at a rate of 1 gram tobacco leaf to 20 grams strawberry leaf. Inoculations with an extract from this mixture, using 10 ml of physiological salt solution per gram of dry powder, produced almost no local lesions on *Nicotiana glutinosa*. (The single instance when a few lesions were produced could have been the result of chance contamination.) The same extract gave color reaction with $FeCl_3$ comparable with a 0.9 % solution of tannic acid.

When the solvent (absolute alcohol or chloroform) had been brought to a rapid distillation, the condensor was momentarily separated from the extraction unit, and a cellulose extraction thimble containing the tobacco-strawberry mixture was inserted. When very small quantities of material were used, a more frequent washing was achieved by filling the extraction chamber part way with sterile glass beads, leaving just enough space so the plant material was covered before the solvent siphoned into the distillation flask. If this was done, a small amount of spun glass was first added to the chamber to prevent a glass bead from blocking the entrance to the siphon tube.

Although tests with $FeCl_3$ indicated that all tannins could be removed from raspberry tissues in 20 minutes, strawberry tissues in 30 minutes, and tea tissues in 30–35 minutes, the extractions with alcohol were carried on for 20, 40, and 60 minutes, and with chloroform for 15, 30, and 45 minutes. At the end of the period the extraction thimble was removed from the apparatus and the powdered leaf mixture was dried rapidly in a vacuum in a desiccator containing P_2O_5 if alcohol was used as the solvent. When chloroform was used, the mixture dried very rapidly in the air and was then transferred to the desiccator.

When all lots had been dried, 1 gram of each was ground in a mortar with 10 grams physiological salt solution, allowed to stand for 6 hours at 20 °C, then centrifuged at 7000 r.p.m. for 20 minutes. The natant solutions were then tested for infectivity and serological activity.

Detached leaves of *Nicotiana glutinosa* were inoculated with the above extracts, using dilutions of 1–100, 1–1000, and 1–10,000. Inoculated leaves were stored in moist chambers under continuous illumination and a temperature of approximately 20 °C for 48 hours, after which the numbers of local lesions were determined. In subsequent tests with potato virus-X, *Gomphrena globosa* leaves were inoculated with dilutions of 1–10, 1–100, and 1–1000, and counts were made after 7 days.

In all serological tests micro-drops were placed on Formvar treated plates and covered with paraffin oil to prevent drying (2). Each extract was tested at dilutions of 1/10, 1/30, 1/90, 1/270, 1/810, and 1/2430. Suitable checks with normal serum and with extract from healthy White Burley tobacco were maintained in all cases. Precipitation reactions were determined after 24 hours at approximately 20 °C.

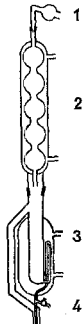

Fig. 1. Modified Soxhlet extraction apparatus, showing $CaCl_2$ trap (1), extended condensor (2), water jacket around extraction chamber (3), and test cock on siphon tube (4).

Gewijzigd Soxhlet-apparaat voor de extractie van looistoffen uit virushoudend plantemateriaal.

TABLE 1. Effect of tannin extraction with absolute alcohol or chloroform on infectivity of tobacco mosaic virus

Tabel 1. Effect van de extractie der looistoffen met absolute alcohol of chloroform op het infectievermogen van tabaksmozaïekvirus

Inoculum from tobacco-strawberry tissue mixture after removal of tannins with *Inoculum van een mengsel van tabaks- en aardbeiblad na verwijdering van de looistoffen met*	Dilution of inoculum *Verdunning van inoculum* 10^{-3}	10^{-4}	10^{-5}
	Number local lesions per leaf[1]) *Aantal vlekjes per blad*[1])		
Alcohol for 20 minutes	132	81	45
„ „ 40 „	109	65	10
„ „ 60 „	218	101	12
Chloroform for 15 minutes	181	59	17
„ „ 30 „	254	99	60
„ „ 45 „	189	94	30
Control-Tannins not extracted *Controle-Looistoffen niet verwijderd*	2	0	0
Control-No strawberry tissue *Controle-Zonder aardbeiblad*	239	120	49

[1]) Average of 4 replications.
Gemiddelde van 4 herhalingen.
Decimals have been omitted.
Decimalen zijn weggelaten.

TABLE 2. Effect of tannin extraction with absolute alcohol or chloroform on serological activity of tobacco mosaic virus [1])

Tabel 2. Effect van de extractie der looistoffen met absolute alcohol of chloroform op de serologische activiteit van tabaksmozaïekvirus [1])

Extracts from tobacco-strawberry tissue mixture after removal of tannins with *Extracten van een mengsel van tabaks- en aardbeiblad na verwijdering van de looistoffen met*	Dilution of extract[2]) *Verdunning van het extract*[2]) 1/10	1/30	1/90	1/270	1/810	1/2430
	Strength of reaction expressed in numbers[3]) *Sterkte van de reactie in een getal uitgedrukt*[3])					
Alcohol for 20 minutes	16	13	9	4	1	–
„ „ 40 „	16	13	7	–	–	–
„ „ 60 „	16	16	7	2	–	–
Chloroform for 15 minutes	16	16	9	7	2	1
„ „ 30 „	16	16	8	7	3	1
„ „ 45 „	16	16	12	6	2	–
Control – No strawberry tissue *Controle – Zonder aardbeiblad*	16	16	16	9	4	1

[1]) Average of 4 replications.
Gemiddelde van 4 herhalingen.
[2]) Serum dilution 1/10 throughout.
Serumverdunning steeds 1/10.
[3]) Smallest observable reaction 1; very strong reaction 16 (See text).
1: reactie nog net zichtbaar; 16: zeer sterke reactie.
Decimals have been omitted.
Decimalen zijn weggelaten.

TABLE 3. Effect of tannin extraction with absolute alcohol or chloroform on infectivity of potato virus-X

Tabel 3. Effect van de extractie der looistoffen met absolute alcohol of chloroform op het infectievermogen van aardappel-X-virus

Inoculum from tobacco-strawberry tissue mixture after removal of tannins with *Inoculum van een mengsel van tabaks- en aardbeiblad na verwijdering van de looistoffen met*	Dilution of inoculum *Verdunning van inoculum* 10^{-1}	10^{-2}	10^{-3}
	Number local lesions per leaf[1]) *Aantal vlekjes per blad*[1])		
Alcohol for 20 minutes	14	4	0
„ „ 40 „	11	3	2
„ „ 60 „	10	1	1
Chloroform for 15 minutes	16	6	3
„ „ 30 „	11	7	1
„ „ 45 „	14	4	2
Control – Tannins not extracted *Controle – Looistoffen niet verwijderd*	0	0	0
Control – No strawberry tissue *Controle – Zonder aardbeiblad*	17	8	3

[1]) Average of 4 replications.
Gemiddelde van 4 herhalingen.
Decimals have been omitted.
Decimalen zijn weggelaten.

TABLE 4. Effect of tannin extraction with absolute alcohol or chloroform on serological activity of potato virus-X[1])

Tabel 4. Effect van de extractie der looistoffen met absolute alcohol of chloroform op de serologische activiteit van aardappel-X-virus[1])

Extracts from tobacco-strawberry tissue mixture after removal of tannins with *Extracten van een mengsel van tabaks- en aardbeiblad na verwijdering van de looistoffen met*	Dilution of extract[2]) *Verdunning van het extract*[2]) 1/10	1/30	1/90	1/270	1/810
	Strength of reaction expressed in numbers[3]) *Sterkte van de reactie in een getal uitgedrukt*[3])				
Alcohol for 20 minutes	14	9	4	1	–
„ „ 40 „	15	7	4	–	–
„ „ 60 „	11	9	3	1	–
Chloroform for 15 minutes	16	16	7	4	1
„ „ 30 „	16	14	9	2	–
„ „ 45 „	13	12	6	4	1
Control – No strawberry tissue *Controle – Zonder aardbeiblad*	16	16	9	8	1

[1]) Average of 4 replications.
Gemiddelde van 4 herhalingen.
[2]) Serum dilution 1/10 throughout.
Serumverdunning steeds 1/10.
[3]) Smallest observable reaction 1; very strong reaction 16 (See text).
1: reactie nog net zichtbaar; 16: zeer sterke reactie.
Decimals have been omitted.
Decimalen zijn weggelaten.

In reading the serological tests the smallest visible reaction was given the numerical value of 1, a moderate precipitate a value of 4, a heavy precipitate a value of 9, and a very heavy precipitate a value of 16. Since the precipitates observed extended in a horizontal plane, the squares of the numbers 1 to 4 respectively present a more accurate interpretation of the strength of the reaction than the numbers themselves.

The results indicate that with a stable virus like tobacco mosaic virus, there is no appreciable loss in either infectivity or serological activity as a result of extraction period of as much as an hour with absolute ethyl alcohol or 45 minutes with chloroform (Tables 1 and 2). Tests using tobacco leaves infected with potato virus-X showed correspondingly slight losses of virus infectivity or serological activity (Tables 3 and 4).

The modification of the Soxhlet extraction apparatus seems to be a satisfactory device for the removal of tannins from plant tissues infected with certain relatively stable viruses.

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SAMENVATTING

Sommige plantesoorten bevatten looistoffen, waarvan wordt aangenomen, dat zij de overbrenging met sap van in deze plantesoorten voorkomende virussen verhinderen. In dit artikel wordt een methode beschreven, waarmee deze looistoffen vooraf uit plantemateriaal kunnen worden verwijderd. Het looistofbevattende blad wordt, na snel diepgevroren en gedroogd te zijn, in een gewijzigd Soxhlet-apparaat (Fig. 1) met alcohol of chloroform uitgetrokken. Aldus scheidt men de looistoffen kwantitatief van het virus.

Het effect van de extractie der looistoffen of de virusactiviteit werd nagegaan door mengsels van tabaksbladeren, respectievelijk geïnfecteerd met tabaksmozaïekvirus en aardappel-X-virus, en aardbeibladeren volgens de beschreven methode van looistoffen te ontdoen en daarna het infectievermogen van deze virussen of *Nicotiana glutinosa*, respectievelijk *Gomphrena globosa*, te toetsen (Tabellen 1 en 3). Tevens werd de serologische activiteit van de aldus van tannine bevrijde virussen bepaald (Tabellen 2 en 4).

Uit de verstrekte gegevens blijkt, dat het tabaksmozaïekvirus en het aardappel-X-virus nagenoeg zonder verlies aan infectievermogen of serologische activiteit uit het mengsel met aardbeiblad kunnen worden geïsoleerd.

LITERATURE

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