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The long-term preservation of potato virus Y and watermelon mosaic virus in liquid nitrogen in comparison to other preservation methods

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Abstract

Samples of watermelon mosaic virus (WMMV), consisting of clarified sap of diseased squash plants, were found to be very infective after more than 4 years of storage if the samples had been deep frozen at -18 °C, and to have lost most of their infectivity within 4 years of preservation by freeze drying and subsequent storage of the samples at 4 °C.
Potato virus Y (PVY) inocula, consisting of samples of clarified sap of diseased red peppers, lost their infectivity in less than 4 years if the samples had been deep frozen at -18 °C but retained their infectivity for more than 4 years if the samples had been freeze dried and stored at 4 °C.
A decrease in infectivity of both viruses in the deep frozen at -18 °C and freeze dried inocula could be observed even during the first months of storage. However, samples of clarified sap stored in or over liquid nitrogen maintained their activity for at least 22 months for WMMV and 32 months for PVY with no indication of a decrease in infectivity of the viruses. Storage in or over liquid nitrogen. seems therefore a very promising long-term preservation method for plant viruses.

Introduction

While searching for a virus preservation method which would provide a standard inoculum for routine trials over several months, we compared three techniques: 1) deep freezing at -18 °C, 2) freeze drying and 3) storage in liquid nitrogen at -196 °C in the liquid phase or at -150 °C in the vapour phase. Since literature on the latter, most recent and least known technique is scarce, it scemed useful to report our observations.

Materials and methods

A common strain of potato virus Y (PVY) and watermelon mosaic virus (WMMV) were isolated by Dr J. N. Simons, Ciba-Geigy Co. Vero Beach, in the field in Florida (USA). Their identity was subsequently determined by host range studies and serology. PVY was isolated from red pepper (*Capsicum annuum*) and WMMV from squash (*Curcurbita pepo*). They were subsequently propagated in red peppers (*Capsicum annuum* cv. California Wonder) and squash (*Curcurbita pepo* cv. Early White Bush Scallop) respectively, by mechanical and/or aphid inoculation of the host plants.
For preservation trials leaves of diseased plants were collected about two weeks after inoculation of the host plants, triturated for three minutes in a mixer (Virtis) at 3000 rpm in 10 times (PVY) or in 5 times (WMMV) the amount (w/v) of phosphate buffer at pH 7 (0.041 M Na_2HPO_4 + 0.028 M KH_2PO_4). The crude sap was clarified by filtering though filter cloth and

by low speed centrifugation. The resulting supernatant was then distributed in equal amounts into appropriate containers. No additives were used as virus preservative during storage.
The clarified sap was deep-frozen at -18 °C in 10 ml amounts in 20 ml test tubes, which were closed with cotton-wool plugs or freeze-dried in 3 ml amounts in 5 ml penicilline bottles and subsequently stored at 4 °C or stored in 1.5 ml amounts in 2 ml plastic tubes with a screw cap in or over liquid nitrogen at -196 °C or about -150 °C. A WKF L2 lyophilisation apparatus was used in which the samples were prefrozen at -40 °C before the vacuum was applied. The vacuum of the bottles was checked with a high frequency vacuum tester (model T 1 of Edward High Vacuum Ltd., Crawley, Sussex).
For retesting we normally used 10 ml of deep-frozen sap at - 18 °C, 4 bottles of freeze dried sap, and two 1.5 ml samples of the sap, stored in or over the liquid nitrogen. The bottles with freeze dried material were filled with 3 ml of bidistilled water before use. The samples kept at -18 °C and in or over liquid nitrogen, respectively, were immersed in water at about 15 °C to reobtain the crude clarified sap. We made no distinction between samples stored in the liquid phase at -196 °C or in the vapour phase at about -150 °C of the boiling nitrogen. These inocula were then diluted with phosphate buffer pH 7 and each dilution was tested on 20 plants of the same host cultivar as used for propagation of the viruses.

Results

The inocula were originally prepared to serve as standard inoculum for routine tests. Therefore, they were tested regularly during the first months of the preservation time. Later they were only tested at irregular intervals of several months or even years.
lt can be seen from Table 1 that PVY from red peppers can be preserved for 2-4 years deep frozen at -18 °C. However, freeze-drying is superior over deep freezing at -18 °C, whereas preservation in or over liquid nitrogen is the most promising of the three methods.
Table 2 shows that deep freezing at -18 °C is obviously a better method of preservation for WMMV than freeze-drying but the infectivity results at the 1: 1000 and 1: 500 dilution show that storage in or over liquid nitrogen, here too, is the most promising.
We attribute the variation in preservation time between the inocula stored in the same way to differences in the initial concentration of virus in the clarified crude sap. Unfortunately, our infectivity data of the control inoculations at the beginning of the preservation period are not sufficient to support this statement but the fact that the inocula lose their infectivity faster the more they are diluted, proves that the infectivity almost certainly depends on the initial virus concentration, although this is perhaps not the only factor of importance.
The deep-frozen at -18 °C and freeze-dried inocula, which were tested whithin a month after the beginning of the preservation period showed, in general, a loss of infectivity when compared to the fresh clarified sap. Although they could all be used as standard inocula during a few or even several months, a decreasing infectivity could be observed even during these first months of preservation. The decrease in infectivity continued, resulting in a total loss of infectivity of most inocula within 4 years.

For Fig. 1 and Fig 2: see end of publication.

Table 1. Retention of infectivity during long-term preservation of potato virus Y.

Method of preservation of the clarified crude sap	Deep freezing at -18 °C	Freeze drying and storage ofthe samples at 4 °C			Storage in or over liquid nitrogen	
Number of inocula evaluated	7	1	1	1	1	1
Date of inoculum production	1971-1972	13-12-71[3]	2-2-73	19-2-75	8-3-73	9-10-74
Dilution tested:						
1:10	24-48[1]	52(40)[2]	36(10)	11(95)	32(100)	16(100)
1:100	12-24	52(15)	18-33	11(85)	32(100)	16(95)
1:1000	2-6	52(5)	0-1	11(40)	32(30)	16(60)
1:10.000						16(10)

[1]) Time lapse in months during which total loss of infectivity of the inocula occurred.
[2]) Minimal time of survival of the virus in the inocula in months with the percentage of infection on 20 plants at the last test in parentheses.
[3]) Day - month - year.

Tabel 1. Behoud van infectiositeit gedurende bewaring op lange termijn van aardappelvirus Y.

Table 2. Retention of infectivity during long-term preservation of watermelon mosaic virus.

Method of preservation of the clarified crude sap	Deep freezing at -18 °C				Freeze drying and storage of the samples at 4 °C		Storage in or over liquid nitrogen
Number of inocula evaluated	1	1	1	2	3	1	1
Production date of inocula	14-1-72[3]	4-8-71	18-5-71	25-5-72	13-6-72	2-3-72	5-4-73
				28-4-72	11-12-73		
					21-4-73		
Dilutions tested							
1:5	51(90)	56(100)	57(80)[2]	36-48[1]	24-42	47(10)	22(100)
1:10	51(95)	56(100)	57(80)		12-28	31-39	22(100)
1:100	51(30)	56(55)	57(20)	36-48	8-24	21-31	22(100)
1:500							22(80)
1:1000	5(60)	17(20)	7(40)	1-12	1-3	17-31	

1, 2, 3: For explanation see Table 1.

Table2. Behoud van infectiositeit gedurende bewaring op lange termijn van Watermeloenmozaïekvirus.

Fig. 1 shows that nos loss of infectivity could be detected in the samples of WMMV stored in or over liquid nitrogen over a period of 22 months. From the last test of PVY in November 1975 at a dilution of 1:1000 (Fig. 2) it might be concluded that a slight loss of infectivity of the PVY samples in or over liquid nitrogen occurred after a preservation period of 32 months. However, all samples of other PVY preservation trials which were tested on the same day and three months later also showed considerably lower infectivity in November 1975 than in March 1976. Therefore, we attribute the relatively low infection level of this last test to factors other than to loss of infectivity of the virus, namely to a lower susceptibility of the test plants in November 1975 than in March 1976 probably under the influence of less favourable growing conditions in November in the greenhouse. Unfortunately, no samples for further testing are left of both inocula, PVY and WMMV, stored in or over liquid nitrogen.

Discussion

The differences in preservation characteristics shown by the PVY and WMMV deepfrozen (-18 °C) and freeze dried inocula must be considered as a result of the differences in virus and host plant properties.
As far as we know this is the first report on long-term preservation of WMMV. An exact comparison of our own PVY preservation results by deep freezing and lyophilization with data previously published was very difficult because of differences in host plants and virus strains used, differences in the freeze-drying technique (with or without additives in the clarified sap), preservation time and test methods. Tobacco, used as PVY source plant in preservation trials (deep freezing, at - 18 °C and lyophilization) by several authors (Yamaguchi, 1964; De Bokx, 1964; Bos, 1969; Hollings and Stone, 1970; Purcifull et al., 1975) might be better than red peppers, since it is known that leaves contain inhibitors of virus infection (Marchoux, 1970; Fischer and Nienhaus, 1973) and Hollings (Loc, sit. Hollings and Stone, 1970) observed that inhibitors of virus infection may affect the virus when concentrated during lyophilisation. Purcifull et al. (1975) obtained, nevertheless, satisfactory results with PVY containing freeze dried sap of red pepper without additives, but the storage period after which he tested was only 3 months. Hollings and Stone (1970) report on samples of freeze dried sap of *Nicotiana glutinosa* that contained active PVY (C strain) after more than 10 years of storage and of a normal strain of PVY that was still active after 5 years of storage at 4 °C. Yamaguchi (1964) obtained satisfactory results with deep freezing (-18 °C) of PVY in entire tobacco leaves for over a year. De Bokx (1964) reports that a rapid decrease in infectivity of deep frozen clarified sap of tobacco infected with PVY^N started immediately after the beginning of the preservation period.
Chemical dehydration of virus containing tissue, a virus preservation method developed by Mckinney (Mckinney et al. 1965), proved to be as good or even better for the long-term preservation of PVY and several other viruses (Bos, 1969) than freeze drying of clarified sap, but a decrease in infectivity still occurred with PVY and other viruses. Our data presented on the preservation of PVY and WMMV in or over liquid nitrogen indicate that no loss of infectivity of the samples occurred over the preservation period investigated.
Mckinney et al. (1961) obtained their promising results with shortterm storage of 6 labile viruses in liquid nitrogen and since then only Rochow et al. (1976) have reported on the conservation of concentrated preparations of barley yellow dwarf virus for up to four years under liquid nitrogen. Their work, done in collaboration with the American Type Culture Collection (1972), who accepted storage of plant viruses under liquid nitrogen as long-term preservation method, also did not indicate any loss of activity of the virus tested. Our results and those of Rochow et al. (1976) support the assumption that storage in or over liquid nitrogen is by far the best long-term preservation method for a considerable number of plant viruses.

Samenvatting

De bewaring op lange termijn van aardappelvirus Y en watermeloenmozaïekvirus met behulp van vloeibare stikstof vergeleken met andere bewaarmethoden

Watermeloenmozaïekvirus inocula, bestaande uit helder gecentrifugeerd sap van zieke squashplanten, bleken hun infectievermogen zeer goed te hebben behouden na meer dan vier jaar bewaring bij -18 °C, maar binnen vier jaar grotendeels te hebben verloren na droogvriezen en bewaren bij 4 °C.
Overeenkomstig bereide aardappelvirus Y inocula uit zieke paprikaplanten hadden hun infectievermogen binnen vier jaar verloren na bewaring bij -18 °C, maar konden hun infectievermogen grotendeels behouden gedurende meer dan vier jaar na droogvriezen en bewaring bij 4 °C.

Binnen enkele maanden na het begin der bewaringsperiode kon al een afname van het infectievermogen van beide virussen in de diepgevroren (-18 °C) en drooggevroren inocula worden gevonden. Helder gecentrifugeerde sapmonsters die in of boven vloeibare stikstof bewaard waren, hadden gedurende tenminste 22 maanden voor het watermeloenmozaïekvirus en 32 maanden voor het aardappelvirus Y hun volle infectievermogen behouden. Bewaring in of boven vloeibare stikstof is daarom de beste van de drie getoetste bewaarmethodes voor opslag op lange termijn van beide virussen. Deze methode lijkt ook veelbelovend voor een groot aantal andere virussen.

Acknowledgments

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Fig. 1 shows that nos loss of infectivity could be detected in the samples of WMMV stored in or over liquid nitrogen over a period of 22 months. From the last test of PVY in November 1975 at a dilution of 1:1000 (Fig. 2) it might be concluded that

Fig. 1. Infectivity changes of the watermelon mosaic virus inoculum of 05-04-73 during its 22 months preservation period in and/or over liquid nitrogen.

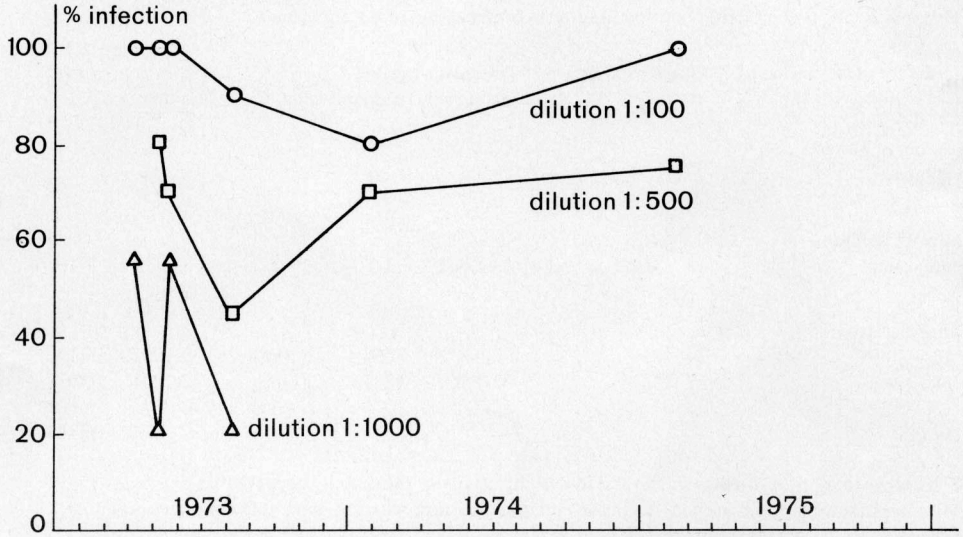

Fig. 1. Veranderingen in het infectievermogen van het watermeloenmozaïkvirus inoculum van 05-04-1973, gedurende de 22 maanden durende bewaarperiode in en/of boven vloeibare stikstof.

Fig. 2. Infectivity changes of the potato virus Y inoculum of 13-08-73 during its 32 months preservation period in and/or over liquid nitrogen.

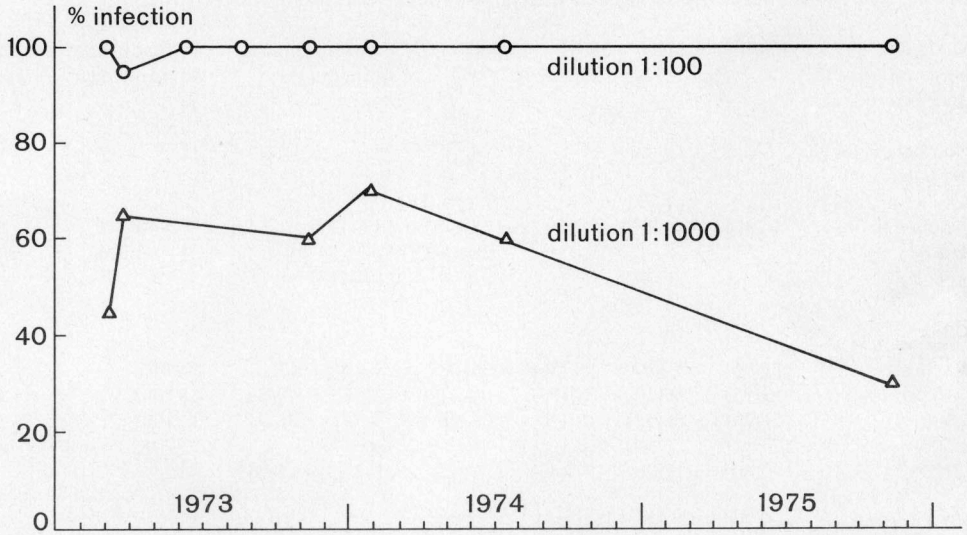

Fig. 2. Veranderingen in het infectievermogen van het aardappelvirus Y-inoculum van 13-08-1973, gedurende de 32 maanden durende bewaarperiode in en/of boven vloeibare stikstof.