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CRYOPRESERVATION OF TOSPOVIRUSES

Angela Yordanova[1*], Dimitrinka Christova[2] and Todor Donev[1]

[1]National Bank for Industrial Microorganisms and Cell Cultures, 1113 Sofia, P.O. Box 239, Bulgaria;
[2]Plant Protection Institute, 2230 Kostinbrod, Bulgaria

Summary

Investigations for conservation in liquid nitrogen of tospoviruses under the guise of infected leaf material and plant sap were performed. The protecting action of four cryoprotecting media was studied. For reading the results an indicator test and ELISA were used. It had been registered that the specific biological properties and the virulent ability were fully preserved in the tospoviruses conserved with 10% dimethylsulphoxid and kept during three years in liquid nitrogen.

Introduction

In Bulgaria the "bronzing" on tobacco had been observed in 1952 by Ivantcheva-Gabrovska in the region of Gotse Deltchev and Sandanski [5]. Kovatchevski described, in the same year, two forms of action on tomatoes - a strong and a weak necrotic one [6]. The cause was the tomato spotted wilt virus (TSWV).

The TSWV possesses isometric particles with a diameter of 70 – 90 nm. The virions are covered with a membrane and contain lipids. The virus has a wide range of hosts and is spread in the temperate and subtropical regions of the world. This is one of the mostly inconstant plant viruses [4].

For a long time the TSWV was concerned to be the single representative of the monotype virus group of genus *Tospovirus*. Today, besides the TSWV, this genus includes also the impatiens necrotic spot virus (INSV) [3]. Some authors referred to the tospoviruses the tomato chlorotic spot virus (TCSV), the groundnut ringspot virus (GRSV) and groundnut but necrosis virus (GBNV), too [2, 10].

Because of the definitely expressed differences in the bronzing symptoms since the time of its observation in Bulgaria, as well as a result of its great economic importance, an investigation on the tospovirus dissemination in the country was started. The collected virus isolates were brought to identification, which required their preservation for a longer period of time. The variants of freeze-drying, successfully used for the conservation of other plant viruses in the National Bank for Industrial Microorganisms and Cell Cultures (NBIMCC) collection, gave insufficient results when lyophilizing TSWV, which quickly lost its infectivity. This necessitated the application of other preservation methods.

The aim of this investigation was to develop reliable variants for long-term-storage of tospoviruses by the use of cryopreservation in liquid nitrogen.

Materials and Methods

Viruses and virus isolates. Isolates from tomatoes, pepper, tobacco and flower cultures, cultivated in greenhouse and in the open, were included in the study (Table 1). The virus isolates were transferred from diseased plants to tobacco (*Nicotiana rustica* or *N. tabacum* cv. 1146). The TCSV, GRSV and INSV were used as referent viruses. The INSV was maintained only on *Impatiens sp*.

Table 1. Investigated tospovirus isolates.

Isolates/ Viruses	Source		Geographic origin	TAS-ELISA[b] (BR - 01 4F2)
	Plant	Symptoms[a]		
1To - 94	tomato (green house)	NS	Trudovets, West Bulgaria	0.599
Za - 95	zanthedeshia (green house)	CRS	Trudovets, West Bulgaria	0.547
317P - 94	pepper (field production)	CRS	Plovdiv, South Bulgaria	0.379
8Hi - 96	hippeastrum (green house)	CRS	Pravets, West Bulgaria	0.447
4GD - 95	tobacco (field production)	NRS	Gotse Deltchev, South-west Bulgaria	0.580
10HK - 95	tobacco (field production)	NRS	Chan Krum, North-east Bulgaria	0.980
20Da - 96	dahlia (field production)	SRS	Negovan, West Bulgaria	0.536
161Za - 96	zanthedeshia (field production)	CS	Negovan, West Bulgaria	0.565
129T - 96	tobacco (field production)	VN	Djebel, South-east Bulgaria	0.471
TCSV - BR-03	tomato (field production)	NRS	Brazil	0.120
GRSV - SA-05	groundnut (field production)	CRS	South Africa	0.123
INSV – NL-07	Impatiens (field production)	NRS	The Netherlands	0.125

[a] symptoms - chlorotic spots (CS), chlorotic ringspots (CRS), necrotic spots (NS), necrotic ringspots (NRS), vein necrosis (VN);
[b] optical density at 405 nm.

Cryoprotectors. 10% glycerine, 10% dimethylsulphoxide (DMSO), 2% polyvinylpyrolidone - 10000 (PVP), 10% sorbitol + 3.6% dextran 40000 were used. The protecting media were prepared in 0.1 M potassium-sodium phosphate buffer, pH 7.2, containing 0.2% sodium sulphite and 0.2% ascorbic acid.

Freezing in liquid nitrogen (LN_2). 1/ Leaves - the infected leaves were cut and then 0.4 g were placed in cryotubes, containing 1.5 ml of protecting medium; 2/ sap - the infected leaves were homogenised with the above mentioned buffer in a ratio 1:2 (w/v). The resulting sap was centrifuged at 10000 rpm for 15 min. The supernatant was mixed with an equal volume of double concentrated protector and then 1.5 ml of this material was poured in cryotubes. All the operations were performed on ice (0^oC).

The cryotubes were equilibrated at 4^oC, as for the sap containing samples the duration was 1 h, while for the leaves containing ones it was 20 h. The freezing was accomplished with a speed of 0.5 - $0.6^oC.min^{-1}$ until a temperature -20^oC had been achieved. Then the samples were transferred to liquid nitrogen and stored there.

The melting of the viruses was performed on water bath at 30^oC.

Indicator test. The infected tissue from diseased plants was homogenised in 0.1 M potassium-sodium phosphate buffer, pH 7.2, containing 0.2% ascorbic acid and 0.2% sodium sulphite, in a ratio 1:1 (w/v). The diagnostic plants were inoculated and in 10 - 15 days the symptoms were read. Twelve plant species belonging to the families *Solanaceae, Chenopodiaceae* and *Amarantaceae*, were used.

After the cryopreservation of the viruses, the diagnostic plants were again inoculated, and the leaves containing samples had been preliminary ground. The symptoms were also read in 10 - 15 days. If there had been a lack of visible symptoms, a blind passage was performed.

Serological tests. Two modifications of ELISA were used for the serological identification of the isolates.

DAS - ELISA was accomplished according to the prescription of the firm "Loewe", Germany, respectively for TSWV, TCSV, GRSV and INRV.

TAS - ELISA was performed according to the following scheme: 1/ polyclonal antibody for TSWV (BR - 01) - 1:1000 in 0.05 M carbonate buffer, pH 9.6; 2/ antigen (plant sap) - 1:100 in PBS-T and 0.5% sodium sulphite; 3/ monoclonal antibody 4F2+[10] - 1:2000 in PBS-T, 2% PVP and 0.2% egg albumin; 4/ alkaline-phosphatase antimouse conjugate - 1:5000 in PBS-T and 2% bovine serum albumin. The extinction was read at 405 nm.

Results and Discussion

The initial tentative investigations had been performed with isolate 1To-94. Two variants of liquid nitrogen freezing were used - leaf material and plant sap, as four cryoprotectors had been applied (Table 2). The infectivity of the samples was tested 24 hours and 3 years after the freezing.

According to the data, the leaf variants had been better conserved. The virus was preserved in this form when protected with glycerine, DMSO and sorbitol/dextran. The last had been routinely used in the NBIMCC for the plant viruses lyophilization [11]. The glycerine, DMSO and PVP had been among the most frequently used protectors for low-temperature preservation of biological materials [1, 8]. PVP did not exhibit a protecting effect during our experiments. Other authors had also successfully applied glycerine, when freezing plant viruses [7, 9].

Table 2. Preservation of tospovirus 1To-94 after liquid nitrogen freezing.

Variant	Protector [b]	Infectivity [a] after	
		Freezing	3 years in LN_2
Leaves	Glycerine	++	++
Leaves	DMSO	++	++
Leaves	PVP	–	–
Leaves	Sorbitol/dextran	++	+
Sap	Glycerine	–	–
Sap	DMSO	+	–
Sap	PVP	–	–
Sap	Sorbitol/dextran	–	–

[a] infectivity - 2 infected plants from 2 inoculated (++), 1 infected plant from 2 inoculated (+), no infectivity (–);

[b] abreviations as in text.

The results, achieved with glycerine and DMSO were comparable, but when few-times repetition had been performed, a better protecting effect of DMSO was observed. This cryoprotector had been chosen for the further investigations on the tospoviruses preservation.

Nine virus isolates were studied (Table 1). The characteristics, detected on the initial plants, varied too much between the different botanical species, but the chlorotic and necrotic spots and rings did predominate. It had been determined, by the use of TAS - ELISA that all the Bulgarian isolates belonged to the TSWV (Table 1).

The nine quoted TSWV isolates, as well as the referent TCSV, GRSV and INSV viruses were conserved in liquid nitrogen. The variant of leaves protected with 10% DMSO was used. All the viruses included in the investigation did keep their infectivity. The biological properties were compared before and after the freezing. The symptoms, induced by the Bulgarian isolates and the referent viruses on the diagnostic plants, did also characterise with a great variety, but were typical for the tospoviruses. Local symptoms were observed on *Petunia hybrida, Chenopodium album, Ch. amaranticolor, Ch. foetidum* and *Ch. quinoa*. In *Datura stramonium, Gomphrena globosa, N. benthamiana, N. clevelandii, N. glutinosa, N. rustica* and *N. tabacum* cv. 1146 the infection was local and systemic.

By testing on tomatoes, it had been established that the tomato isolates were extremely virulent for this culture. As concerning the tobacco isolates, 10HK-95 from North Bulgaria was quite more virulent for the tomatoes, when compared with the 4GD 95, that originated from South Bulgaria (Table 1). The first one formed distinctly exhibited necrotic spots on the leaves and necrosis on the stem, while the second caused mosaic and chlorotic rings.

These specific biological properties of the virus isolates and the referent viruses are preserved after a three-year-period of conservation in liquid nitrogen. No changes, concerning the virulent ability, are observed.

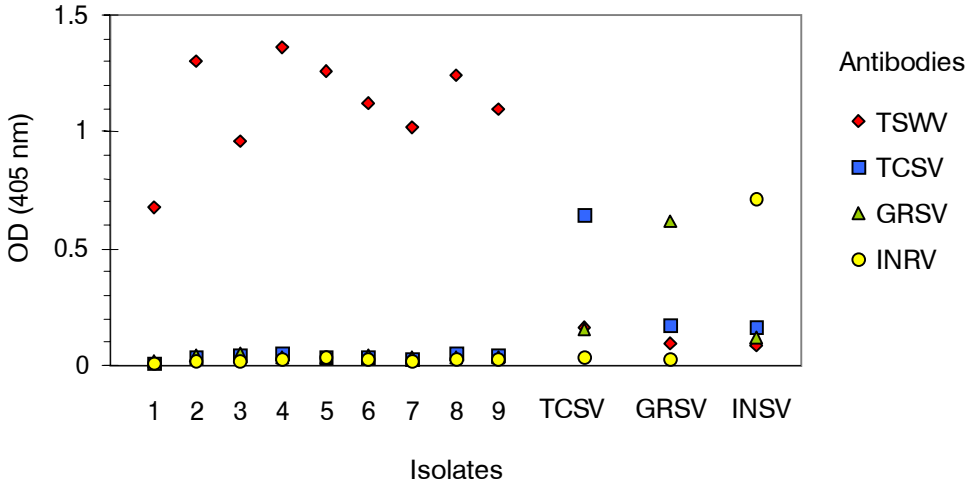

Fig. 1. DAS-ELISA of tospoviruses after cryopreservation in liquid nitrogen. Isolates 1 - 9 according to Table 1.

After the revitalisation of the frozen in liquid nitrogen viruses, the samples were also tested with DAS-ELISA (Fig. 1). The individual representatives of genus *Tospovirus* distinctly differentiated. The belonging of the Bulgarian isolates to the TSWV was confirmed.

The results that have been achieved, show that the studied viruses, protected with DMSO - 10%, completely preserve their infectivity and biological characteristics after a freezing at minus 196°C.

This gives us a reason for applying the reported methodology in our routine work for liquid nitrogen conservation and preservation of tospoviruses.

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