Floriculture and Ornamental Biotechnology ©2012 Global Science Books

Virus Eradication in Narcissus and Tulip by Chemotherapy

Dariusz Sochacki* • **Małgorzata Podwyszyńska**

Research Institute of Horticulture, Konstytucji 3 Maja 1/3, 96-100 Skierniewice, Poland

Corresponding author: * **dariusz.sochacki@inhort.pl**

ABSTRACT

The aim of the research was to obtain virus-free stock plant material of several cultivars and breeding clones of narcissus (*Narcissus* L.) and tulip (*Tulipa* L.) by *in vitro* culture using chemotherapy with ribavirin. Virus indexing by ELISA was done several times to detect the most important viruses infecting tulips and narcissus. Genotypes of both crops, totally or heavy infected by viruses, were subjected to chemotherapy in a few experiments. The results of virus eradication showed that none of the three concentrations of ribavirin (12.5, 25 and 50 mg l^{-1}) did result in death. However, shoot regeneration and growth on the medium with the highest concentration of ribavirin was significantly retarded, both for tulips and narcissus. Chemotherapy joined with further consecutive virus indexing and roguing the virus-suspected plantlets of tulip S2 and S3 enabled selection of virus-negative plants. The results of chemotherapy obtained for the new tulip cultivars (S2, S6 and S7) in the next experiment were very promising. The ribavirin treatment resulted in virus eradication from the newly forming shoots. In turn, in the old cultivars (E and F) and one breeding line (P7), whose shoots were totally infected, chemotherapy appeared to be ineffective. Virus eradication was unsuccessful for all treated plantlets of narcissus 'Lajkonik' infected by *Narcissus mosaic virus* and potyviruses in two experiments. Virus eradication for breeding clone 0.985T infected by *Narcissus latent virus* and potyviruses was successful in 27 among the 80 plantlets which started the experiment. The results leads to conclusion that the effect of ribavirin depends on the concentration of this antiviral agent, kind of virus and their concentration in plant tissue and on the genotype of the plant.

Keywords: ELISA, *in vitro*, ribavirin, virus-free plant

Abbreviations: CMV, *Cucumber mosaic virus*; **ELISA**, enzyme linked immunosorbent assays; **DAS-ELISA**, double antibody sandwich ELISA; **LSV**, *Lily symptomless carlavirus*; **NDV**, *Narcissus degeneration potyvirus*; **NLSYV**, *Narcissus late seasons yellows potyvirus*; **NLV**, *Narcissus latent carlavirus*; **NMV**, *Narcissus mosaic potexvirus*; **NWSV**, *Narcissus white streak potyvirus*; **NYSV**, *Narcissus yellow stripe potyvirus*; **PBS**, phosphate buffer saline; **TBV**, *Tulip breaking potyvirus*; **TNV**, *Tobacco necrosis necrovirus*; **TRV**, *Tobacco rattle tobravirus*

INTRODUCTION

Viral diseases can drastically affect the yield and quality of tulip and narcissus bulbs and flowers, sometimes resulting in total crop loss. Commercial bulb reproduction involves vegetative propagation. Vegetative propagation can lead to the accumulation of viruses and their spreading by daughter bulbs. For tulip, 22 viruses have been reported (Mowat 1995). The most common ones causing serious symptoms include: *Tulip breaking potyvirus* (TBV), *Lily symptomless carlavirus* (LSV), *Cucumber mosaic cucumovirus* (CMV), *Tobacco necrosis necrovirus* (TNV), and *Tobacco rattle tobravirus* (TRV). At least 24 viruses have been reported for narcissus (Brunt 1995; Asjes 1996). Ten of them occur only in restricted geographical locations, the others are spread world-wide. Both crops can be infected by more than one virus at the same time. However, mixed infections occur in narcissus more often than in tulips. The most dangerous for narcissus are: *Narcissus yellow stripe potyvirus* (NYSV), *Narcissus white streak potyvirus* (NWSV), and *Narcissus degeneration potyvirus* (NDV) (Hanks 1993). The last one is known only to occur in *Narcissus tazetta* (Brunt 1980). Very common are also: *Narcissus late seasons yellows potyvirus* (NLSYV), *Narcissus latent carlavirus* (NLV) and *Narcissus mosaic potexvirus* (NMV).

When propagating geophytes, it is essential to start with healthy, virus-free material. Micropropagation of selected virus-free plants and the elimination of viruses during *in vitro* culture are the best methods to ensure that the breeding and planting stock is healthy. In case of totally infected cultivars, it is possible to eliminate viruses and obtain healthy material by application of chemotherapy and/or thermotherapy combined with *in vitro* techniques (Hansen and Lane 1985; Spiegel *et al.* 1993). Within geophytes, positive results using such techniques were obtained in alstroemeria (Hakkaart and Versluijs 1985), gladiolus (Lilien-Kipnis *et al.* 1992; Mangal *et al.* 2003), hyacinth (Asjes *et al.* 1974) and bulbous iris (Van der Linde *et al.* 1988). Elimination of viruses from bulbous plants is, however, very difficult. The most effective method to obtain pathogen-free material is selecting healthy indexed plants e.g. with ELISA and their fast propagation (Spiegel and Loebenstein 1995). Micropropagation is considered as the most potent propagation technique. This method is recommended for nuclear stock production by European and Mediterranean Plant Protection Organization (OEPP/EPPO) for several vegetatively propagated ornamentals, such as carnation, chrysanthemum, begonia, New Guinea hybrids of impatiens, rose, kalanchoe, narcissus and lily (Anonymous 2002). *In vitro* techniques are also used for maintenance of pathogen-free nuclear stocks (Brunt 1985). In our earlier studies, virus-free stock plants of several tulip cultivars were obtained using *in vitro* propagation by testing plant material repeatedly with ELISA against five viruses (TBV, LSV, CMV, TNV and TRV), and selecting virus-negative plants for further propagation (Podwyszyńska *et al.* 2005). It was also shown, that in one of the studied cultivars, 'Giewont', the only TBV-negative shoot clone obtained was derived from the bulb for which the absorbance A_{405} at the initial stage testing was very close to the negative control. Our previous experiments, revealed that TBV detection was more effective in samples taken from leaves than in those

Received: 10 June, 2011. Accepted: 20 December, 2012.

Original Research Paper

derived from scales, the tissues that remain after excision of the flower stem from which fragments are being used for initiating *in vitro* culture (Sochacki and Podwyszyńska 2006). Furthermore, detection of TBV (belonging to potyvirus group) using antibodies specific for TBV was much more reliable than using antibodies universal for the potyvirus group. In some cases only looking for non-infected plants was not enough and additional treatments such the chemotherapy had to be applied.

In narcissus, potyvirus-free plants were obtained in an earlier study from infected bulbs using *in vitro* propagation via adventitious shoot regeneration (Sochacki and Orlikowska 2005). Just as for tulips, obtaining of virus-free narcissus plants by selecting non-infected plants and serious virus indexing was a goal of the recent experiments (Sochacki 2011). Individual virus-negative clones were selected from narcissus donor plants infected with NMV and NLV based on indexing done during micropropagation. The results of above mentioned research showed that micropropagation via adventitious shoot regeneration made it possible to eliminate the viruses from infected narcissus plants. The success depended on the type of virus and its concentration in the plant tissue. In addition, chemotherapy was necessary in the case of totally infected narcissus genotypes.

The aim of presented studies was to obtain virus-free stock plant material of several cultivars and breeding clones of tulip and narcissus by chemotherapy with ribavirin as antiviral compound.

MATERIALS AND METHODS

Plant material

Plants of Polish old and new tulip (*Tulipa* L.) cultivars without symptoms of viral diseases and breeding lines of tulip were used for initiation of *in vitro* shoot cultures. The old cultivars were labeled A to F; the new selections S1 to S8, and the breeding clones P1 to P8. *In vitro* shoots of previously established cultures of two narcissus (*Narcissus* L.) genotypes – the Polish cultivar 'Lajkonik' and the Polish breeding clone 0.985T - were used for experiments with virus eradication. The shoot cultures were established and multiplied via adventitious shoot regeneration, following protocols developed in previous studies on tulip (Podwyszyńska and Marasek 2003) and narcissus (Sochacki and Orlikowska 2005). Shoot cultures of tulip were started from flower stem explants isolated from cooled bulbs in January 2004 and 2005, shoot cultures of narcissus were started in 2008. As initial explants of narcissus, fragments of single scales connected by a section of the basal plate were used.

Serological tests

Enzyme Linked ImmunoSorbent Assays (ELISA) were used to detect the following viruses: for tulip TBV, LSV, CMV, TNV and TRV and the potyvirus group; for narcissus *Arabis mosaic virus* (ArMV), CMV, NLV, NMV and the potyvirus group. DAS-ELISA (Clark and Adams 1977) was used with specific antibodies for the detection of the LSV, NMV, NLV, TBV, TNV (from Applied Plant Research – Flower Bulbs and Nursery Stock Sector, Lisse, The Netherlands), for the detection of ArMV and TRV (from Loewe Phytodiagnostica, Sauerlach, Germany) and for the detection of the CMV (from the Research Institute of Horticulture, Skierniewice, Poland) (Kamińska *et al*. 2005). DAS-ELISA tests were performed according to the protocols of the producers with minor modifications. The leaves were ground in phosphate buffer saline (PBS), pH 7.4, and all the conjugates were diluted in PBS-T containing 0.1% Tween 20 (1:5, w/v). To detect most viruses of the potyvirus group indirect ELISA with monoclonal antibodies were used (Jordan and Hammond 1986; Jordan 1989; Derks 1992) according to the protocol provided with the commercial kit (Agdia Inc., Elkhart, IN, USA) with minor modifications described earlier (Sochacki and Orlikowska 2005).

Virus-indexing in tulip was performed for donor plants and then repeated several times for micropropagated shoots during the shoot multiplication stage, *viz*., after 5, 6, 9, 12, 14 or 22 months from initiation. Besides, donor plants used for starting *in vitro* cultures in January 2004 were tested only at initial stage, but the donor plants serving as explants source in 2005 were tested twice, during the flowering in spring, and at initial stage.

Each sample was tested in 2-3 replicates. The measurements were carried out with a Multiscan Plus reader (Labsystems, Finland) at 405 nm. The samples were recorded as infected when the value of absorbance (A_{405}) was at least twice as high as the A_{405} value for the negative control (Clark *et al.* 1988). In addition, samples having an A_{405} value that exceeded the negative control by 50-99% were recorded as "virus-suspected" (vs). The virus-infected shoot-clones were removed, except for genotypes that were highly or totally infected. These shoot-clones were subjected to chemotherapy. In such genotypes, the most vigorous shoots, indexed as "virus-suspected" plants were selected and micropropagated for further testing.

Chemotherapy

Tulips

In the first experiment, shoot cultures of the three genotypes (D, P1 and P2) established in 2004 were used. They were totally infected with TBV but were negative for other tested viruses. Ribavirin (virazole, 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) (0.22 μm filter-sterilized) was added to the multiplication medium. The medium is described in previous studies (Podwyszyńska and Sochacki 2010). Shoots were maintained on media containing ribavirin at 12.5, 25, or 50 mg l^{-1} for 10 weeks and subsequently were subcultured two times at two-month intervals on media containing ribavirin at 12.5 mg l^{-1}. The initial number of shoots used for each treatment was 56 (four flasks, each containing four clumps with 3-5 shoots). The total number of shoots obtained after the first subculture with ribavirin was counted to assess phytotoxicity. Shoots were tested for viruses following chemical treatment after the last ribavirin-subculture and another three times as described in **Table 1**.

In the second experiment, started in 2005, treatment with ribavirin at 12.5 mg l^{-1} was given to initial explants derived from virus-positive donor bulbs of 7 genotypes (**Table 3**). Ribavirin concentration was selected on base of the results of the first experiment. All initial explants were subjected to chemotherapy during the period of four months of initial culture. Regeneration capacity was compared between untreated (virus-negative) and ribavirin-treated virus-positive explants. The micropropagated shoots were tested for viruses following chemical treatment at successive micropropagation phases at different terms described in **Tables 4** and **5**.

Narcissus

In the case of the variety 'Lajkonik', almost totally infected with NMV and potyviruses, an experiment on virus eradication using ribavirin was carried out in 2009 and 2010. MS medium with ribavirin at 12.5, 25 or 50 mg l^{-1} (0.22 μm filter-sterilized) was used. Medium without ribavirin was the control. Twenty bulblets from *in vitro* cultures were cut into four parts and each segment was transferred into a 100 ml Erlenmeyer flask with fresh MS medium with a different ribavirin level. The explants were grown on medium with ribavirin for 10 weeks. After 10 weeks, the plantlets were virus-tested by ELISA. The same cultures of 'Lajkonik' with the same concentrations of ribavirin were used to repeat the experiment in 2010, because of any successful results obtained from the first one. Similar experiments with the same concentrations of ribavirin were carried out in 2010/11 for Polish breeding clone 0.985T infected by NLV and potyviruses.

RESULTS

Tulips

In the first year of the studies, 2004, ELISA indexing revealed that the donor plants were highly infected with TBV (89.1%) (**Table 1**). TNV, CMV, LSV and TRV occurred sporadically. It is noteworthy that these donor plants

Table 1 Detection of viruses by ELISA at succeeding phases of micropropagation process in tulip plant material of the genotypes which were partly infected with viruses (S1, S2 and S3) and totally infected (D, P1 and P2). Virus-free plant material was gradually selected based on ELISA. The totally infected genotypes were subjected to chemotherapy during the period of six months, between the 2nd and 3rd indexing term. *In vitro* shoot cultures were initiated in 2004.

Genotype	Term of indexing	No. of indexed donor plants or shoot clones or shoot clumps*	No. of infected donor plants or shoot clones or shoot clumps*						No. of virus-negative donor plants or shoot clones or shoot clumps*
			TBV	TNV	LSV	CMV	TRV	Poty-viruses	
S1	1st	17	7+3 vs	0	0	0	1 vs	1 vs	5
(new cultivar)	2nd	10	1+2 vs	0	0	0	0	0	7
	3rd	9	0	nt	nt	nt	nt	0	9
	4th	144	3+3 vs	nt	nt	nt	nt	nt	138
S2	1st	14	4+10 vs	0	0	0	0	8 vs	0
(new cultivar)	2nd	10	8 vs	0	0	0	0	8 vs	2
	3rd	10	0	nt	nt	nt	nt	0	10
	4th	72	0	nt	nt	nt	nt	nt	72
S3	1st	17	12+5 vs	0	1 vs	3 vs	2+2 vs	6 vs	0
(new cultivar)	2nd	5	0	0	0	0	0	2 vs	3
	3rd	5	0	nt	nt	nt	nt	0	5
	4th	35	0	nt	nt	nt	nt	nt	35
D	1st	10	10	0	0	0	0	9	0
(old cultivar)	2nd	8	8	0	0	nt	nt	8	0
	3rd	8	8	0	0	nt	nt	0	0
	4th	15	14	1	0	2+1 vs	1	nt	1
	5th	38	24+8 vs	0	0	4 vs	1 vs	nt	6
	6th	23	13+7 vs	2 vs	0	3+2 vs	0	nt	2 (decayed)
P1	1st	3	3 vs	1 vs	0	0	0	3 vs	0
(breeding clone)	2nd	3	2+1 vs	1 vs	0	nt	nt	1+2 vs	0
	3rd	12	1+3 vs	0	0	nt	nt	0	9
	4th	32	0	0	0	0	0	nt	32
	5th	nt	nt	nt	nt	nt	nt	nt	nt
	6th	30	0	2 vs	0	2+2 vs	0	nt	26
P2	1st	3	3	1 vs	1 vs	1	1	0	0
(breeding clone)	2nd	3	0	0	0	nt	nt	0	3
	3rd	12	0	0	0	nt	nt	0	12
	4th	56	0	0	0	0	0	nt	56
	5th	nt	nt	nt	nt	nt	nt	nt	nt
	6th	38	0	0	0	0	0	nt	38
			Total % of virus positive and vs plants						
	1st	64	89.1	1.5	3.1	6.3	9.4	42.2	
	2nd	39	56.4	1.5	0	-	-	53.8	
	3rd	63	17.5	0	0	-	-	-	
	4th	386	9.8	0	0	1.0	0.3	nt	

*Terms of indexing: 1) at initial stage (donor plants), 2) after 6 months and 3) after 12 months (samples were taken from each shoot clone, each originated from individual donor plant), 4) after 18 months (single sample were collected from each pot), 5) after 19 months, only for cultivar D (samples collected from each shoot clump); 6) after 22 months, for genotypes D, E and F (samples collected from each shoot clump)
nt – not tested
vs - "virus-suspected" plants

Table 2 Effect of ribavirin on adventitious shoot regeneration in tulip genotypes totally infected. *In vitro* shoot cultures were initiated in 2004.

Genotype	Ribavirin (mg l^{-1})	No. of shoots*
D	0	75
	12.5	39
	25	33
	50	10
P1	0	70
	12.5	-
	25	57
	50	30
P2	0	73
	12.5	139
	25	50
	50	37

* No. of shoots obtained after 10-week treatment with ribavirin; the initial number of shoots was 56

Fig. 1 Adventitious shoot cultures of old tulip cultivar 'D' treated with ribavirin. From left to right: control, 12.5, 25 and 50 mg l^{-1}. Ribavirin at higher concentrations caused necrosis of the shoots.

were tested only at initial stage and they were not indexed earlier at active growth during the spring. Two genotypes, D and P2, were totally infected and within the remaining four genotypes, only a few clones were considered as virus-negative or virus-suspected (**Table 1**). From the totally infected genotypes, three, D, P1 and P2 were subjected to chemotherapy. The effect of ribavirin depended on the concentration and the genotype. Regeneration capacity of the old cultivar D was inhibited at 12.5 mg l^{-1}, whereas in the breeding line P1, even a higher number of shoots was obtained at this treatment (**Table 2**). Ribavirin at higher concentrations caused necrosis of many shoots thereby reducing their number (**Fig. 1**). The results of the virus indexing were ambiguous. Independently of the ribavirin concentration, TBV was detected in all samples of the cultivar

Table 3 Regeneration efficiency of initial explants in different tulip genotypes on media without (-) and with addition of 12.5 mg l^{-1} ribavirin (+). *In vitro* shoot cultures were initiated in 2005.

Genotype	No. of donor bulbs		No. of initial explants		% of regenerating explants	
	Ribavirin		Ribavirin		Ribavirin	
	(-)	(+)	(-)	(+)	(-)	(+)
S3	2	2	35	36	74.3	58.3
S4	5	2	66	37	78.8	81.1
S5	1	3	12	55	58.3	29.1
P3	3	3	38	35	78.9	37.1
P4	1	4	24	69	62.5	21.7
E	5	8	65	132	36.9*	34.8
F	2	10	24	159	70.8	25.2

*viruses were detected as early as 9 months after *in vitro* culture initiation

D just after chemotherapy (**Table 1**, 3rd term of indexing). Although, in a few plant samples of this cultivar, for which ELISA performed a few months later, chemotherapy did not reveal TBV. A test performed one year later detected this virus in all samples (data not shown). Instead, in the genotype P2, no viruses were detected at any indexing term, and in genotype P1, 86.7% of tested samples was virus-negative (**Table 1**). In the cultivar S1, only virus-negative explants were selected for further micropropagation, and the occurrence of the virus-positive shoots was sporadic in the later testing terms. In the genotypes S2 and S3, virus-negative plants were absent, but some plants were considered as only virus-suspected and they were further propagated, successively tested, the virus-positive and virus-suspected shoot clones were eliminated, and finally, the virus-negative plants were selected (**Table 1**).

In 2005, more donor plants were indexed as virus negative (26.9%) than in 2004 (5.0%) when the donor plants were tested only once at the initial stage. The virus-negative donor plants in 2005 were selected based on the early ELISA indexing performed during the spring, and testing and selecting was repeated just before *in vitro* cultures initiation. With regard to the still very high virus infection of donor plants in 2005, the *in vitro* cultures were started also from the virus-positive plants. Explants derived from virus-positive plants, incubated for 10 weeks on medium containing 12.5 mg l^{-1} ribavirin exhibited generally lower regeneration capacity (**Table 3**). Results of ELISA showed that from term to term of indexing, the number of the virus-

Table 4 Detection of viruses by ELISA at succeeding phases of micropropagation process in tulip plant material of the genotypes which were partly infected with viruses (S1, S3, S4, S5 and S8) and totally infected (S2, S6 and S7). Virus-free plant material was gradually selected based on ELISA. All the initial explants derived from virus-positive donor plants were subjected to chemotherapy during the period of four months of initial culture. *In vitro* shoot cultures were initiated in 2005.

Genotype	Term of indexing*	No. of tested donor plants or shoot clones or shoot clumps*	No. of donor plants of which explants were treated with ribavirin	No. of infected donor plants or clones or shoot clumps*					No. of virus-negative donor plants or shoot clones or shoot clumps*
				TBV	TNV	LSV	CMV	TRV	
S1	1	10	0	1 vs	0	0	0	0	9
	2	nt		nt	nt	nt	nt	nt	nt
	3	41		0	0	0	0	0	41
	4	66		0	1 vs	0	0	0	65
S2	1	11	11	5+6 vs	0	0	1 vs	2+1 vs	0
	2	nt		nt	nt	nt	nt	nt	nt
	3	25		0	0	0	0	0	25
	4	51		0	0	0	0	0	51
S3	1	5	2	1+2 vs	0	0	0	0	2
	2	nt		nt	nt	nt	nt	nt	nt
	3	13		0	0	0	2 vs	0	11
	4	13		0	0	0	0	0	13
S4	1	7	2	2	0	2 vs	1	1	5
	2	nt		nt	nt	nt	nt	nt	nt
	3	17		2	0	0	0	0	15
	4	53		0	0	0	0	0	53
S5	1	4	2	3	0	0	1 vs	1 vs	1
	2	nt		nt	nt	nt	nt	nt	nt
	3	8		2	1 vs	0	1 vs	1 vs	5
	4	4		0	0	0	0	0	4
S6	1	2	2	2	0	0	0	0	0
	2	nt		nt	nt	nt	nt	nt	nt
	3	2		0	0	0	0	0	2
	4	4		0	0	0	0	0	4
S7	1	5	5	4+1 vs	1+1 vs	0	0	0	0
	2	nt		nt	nt	nt	nt	nt	nt
	3	8		3	0	0	1+1 vs	1+1 vs	4
	4	4		0	0	0	0	0	4
S8	1	5	0	0	0	0	0	0	5
	2	nt		nt	nt	nt	nt	nt	nt
	3	3		0	0	0	2+1 vs	1	0
	4	13		0	0	0	0	0	13
				Total % of infected plant samples					
	1			55.1	2.0	4.1	4.1	10.2	
	2			nt	nt	nt	nt	nt	
	3			1.0	0.9	0.0	6.8	3.4	
	4			0.0	0.5	0.0	0	0	

*Terms of indexing: 1) at initial stage (donor plants); 2) after 5 months (samples were taken from each shoot clone, each originated from individual donor plant); 3) after 9 months (single samples were taken from each container containing shoot cultures); 4) after 14 months (single samples were taken from each container containing shoot cultures).

nt – not-tested

vs - "virus-suspected" plants

Table 5 Detection of viruses by ELISA at succeeding phases of micropropagation process in tulip plant material of the genotypes which were partly infected with viruses(P3, P4, E) and totally infected (P5, P6, P7, P8, G, F). Virus-free plant material was gradually selected based on ELISA. All the initial explants derived from virus-positive donor plants were subjected to chemotherapy during the period of four months of initial culture. *In vitro* shoot cultures were initiated in 2005.

Genotype	Term of indexing*	No. of tested donor plants or shoot clones or shoot clumps*	No. of donor plants of which explants were treated with ribavirin	No. of infected donor plants or clones or shoot clumps*					No. of virus-negative donor plants or shoot clones or shoot clumps*
				TBV	TNV	LSV	CMV	TRV	
P3	1	6	3	1+2 vs	0	0	0	0	3
	2	nt		nt	nt	nt	nt	nt	nt
	3	18		0	0	0	0	0	18
	4	19		0	0	0	0	0	19
P4	1	5	4	1+3 vs	0	0	0	0	1
	2	3		0	0	0	0	0	3
	3	5		0	0	0	0	0	5
	4	7		0	2 vs	0	0	0	5
P5	1	5	5	2+3 vs	0	0	0	1 vs	0
	2	nt		nt	nt	nt	nt	nt	nt
	3	6		0	0	0	0	0	6
	4	11		0	0	0	0	0	11
P6	1	8	8	1+3 vs	7	4+2 vs	0	0	0
	2	7		0	0	0	0	0	7
	3	9		0	0	0	0	0	9
	4	3		0	0	0	0	0	3
P7	1	6	6	3+3 vs	0	0	0	0	0
	2	3		3	0	0	1 vs	0	0
	3	13		9+2 vs	0	0	0	0	2
	4	-		-	-	-	-	-	decayed
P8	1	5	5	3+2 vs	1	0	0	0	0
	2	nt		nt	nt	nt	nt	nt	nt
	3	6		0	0	0	0	0	6
	4	4		0	0	0	0	0	4
G	1	4	4	2+1 vs	3	3	1	2	0
	2	3		0	0	0	0	0	3
	3	2		0	0	0	0	0	2
	4	-		-	-	-	-	-	decayed
E	1	13	8	4+2 vs	2 vs	0	0	1+1 vs	5
	2	3		2+1 vs	0	0	0	0	0
	3	13		10+1 vs	3 vs	0	1+7 vs	0	0
	4	-		-	-	-	-	-	decayed
F	1	12	12	10	0	2 vs	0	0	2
	2	nt		nt	nt	nt	nt	nt	nt
	3	15		8	0	0	0	0	7
	4	-		-	-	-	-	-	decayed
				Total % of infected plant samples					
	1			71.0	26.1	20.3	1.4	7.2	
	2			28.6	0	4.8	4.8	0	
	3			33.3	3.3	0	8.9	0	
	4			0.0	3.9	0	0	0	

*Terms of indexing: 1) at initial stage (donor plants); 2) after 5 months (samples were taken from each shoot clone, each originated from individual donor plant); 3) after 9 months (single samples were taken from each container containing shoot cultures); 4) after 14 months (single samples were taken from each container containing shoot cultures).
nt – not-tested
vs - "virus-suspected" plants

Table 6 Summarized results concerning detection of viruses by ELISA in tulip plant material propagated *in vitro* and gradually selected for virus negative plants based on ELISA. All the initial explants derived from virus-positive donor plants were subjected to chemotherapy during the period of four months of initial culture. *In vitro* shoot cultures were initiated in 2005.

Plant material	No of genotypes	No. of plant samples	No. of infected donor plants or shoot cultures					% virus-negative donor plants or shoot cultures
			TBV	TNV	TRV	CMV	LSV	
Donor bulbs	18	119	63.9	15.1	10.9	5.0	17.0	26.9
Shoot cultures 9 months after culture initiation	18	207	17.9	2.9	1.9	8.2	0.0	77.8
Shoot cultures 14 months after culture initiation	13	208	0	0.5	0	0	0	98.8

positive and virus-suspected (vs) shoot samples decreased (**Tables 4, 5, 6**). After each indexing all virus-positive and virus-suspected shoots were removed. At the third testing term, nine months after culture initiation, viruses were detected both in some treated and untreated shoot-clones. Thus, 77.8% of shoots were considered as virus-negative (**Table 6**). Fourteen months after initiation, except for the three samples, all remaining shoot-clones (98.8%) were virus-negative. One sample of the new cultivar S1 and two samples of the breeding line P4 were considered as virus-suspected and they were eliminated (**Tables 4, 5**). The results of chemotherapy obtained for the new cultivars S2, S6 and S7 are very promising. Before chemotherapy and selection procedure, these cultivars were considered as highly or totally infected. The ribavirin treatment caused the virus eradication from the newly formed shoots, thus, numerous shoots were indexed as virus-negative (**Table 4**). In turn, in the old cultivars (E and F) and the one breeding

Table 7 The selected results of virus eradication in narcissus breeding clone 0.985T using ribavirin; ELISAs performed in 2011

Concentration of ribavirin in the medium [mg l^{-1}]	Description of the probes	The viruses detected in starting material before treatment of ribavirin	Results of ELISA for the plants regenerated on medium with ribavirin				
			ArMV	CMV	NLV	NMV	Potyviruses
0	73	Potyviruses	-	-	-	-	-
	75	Potyviruses	-	-	-	-	-
	77	NLV, Potyviruses	-	-	-	-	-
	77	NLV, Potyviruses	-	-	vs	-	+
	79	NLV, Potyviruses	-	-	-	-	-
	80	Potyviruses	-	-	-	-	+
12.5	90	NLV	-	-	-	-	-
	91 (x3)	Potyviruses	-	-	-	-	-
	91	Potyviruses	-	-	-	-	vs
	92 (x2)	NLV	-	-	-	-	-
	93	NLV, Potyviruses	-	-	-	-	-
	95	NLV, Potyviruses	-	-	-	-	-
	95	NLV, Potyviruses	-	-	-	-	+
	98	NLV	-	-	-	-	-
	98	NLV	-	-	+	-	-
	165	NLV	-	-	+	-	-
25	99 (x2)	NLV, Potyviruses	-	-	+	-	-
	100 (x2)	NLV, Potyviruses	-	-	-	-	-
	102	NLV, Potyviruses	-	-	-	-	-
	104	NLV	-	-	-	-	-
	105	NLV, Potyviruses	-	-	-	-	-
	108	NLV	-	-	-	-	-
	108	NLV	-	-	+	-	+
	109	NLV	-	-	-	-	-
	167	NLV	-	-	vs	-	-
	168 (x2)	NLV, Potyviruses	-	-	-	-	-
50	115	ArMV, NLV, Poty	-	-	-	-	-
	169	NLV, Potyviruses	-	-	-	-	+
	172	NLV, Potyviruses	-	-	-	-	-
	173	NLV, Potyviruses	-	-	-	-	-
	178	NLV, Potyviruses	-	-	+	-	+

Explanations: - negative result; + positive result; vs - virus-suspected

line (P7), whose shoots were totally infected, chemotherapy appeared to be ineffective. Plant material of these infected genotypes had very poor regenerating capacity and finally died (**Table 5**).

All virus-negative shoots of 13 genotypes were further propagated and subsequently, induced for bulb formation. The bulbs were rooted and planted *ex vitro* in an insect-proof tunnel. Four years of observation and indexing did not detect viruses in micropropagated tulips. Each year, leaves of ten plants of each genotype were randomly chosen and tested by ELISA (data not shown).

Narcissus

The results of virus eradication in the cultivar 'Lajkonik' and breeding clone 0.985T using ribavirin showed that none of the three concentrations of ribavirin (12.5, 25 and 50 mg l^{-1}) resulted in dying of explants. However, shoot growth was significantly retarded on medium with the highest concentration of ribavirin. Virus eradication was unsuccessful for all treated plantlets of 'Lajkonik' infected by NMV and potyviruses at both dates of experiment (data not shown). Virus eradication for breeding clone 0.985T was successful in 27 among the 80 plantlets which started the experiment in 2010 and tested in 2011 (**Table 7**). Four virus-negative results were obtained on control medium without ribavirin, 9, 11 and 3 on medium with 12.5, 25 and 50 mg l^{-1}, respectively.

DISCUSSION

The rate of virus elimination depended on the plant genotype, the type of virus and virus concentration. The old tulip cultivar 'D' was infected the most and its regeneration capacity was the lowest. In this cultivar, only a few shoot-clumps were indexed as virus-negative at the last indexing term. In the new cultivars of tulip (S1, S2 and S3) and the breeding lines (P1 and P2), almost all indexed shoot clumps were recorded as virus-negative after 18-month micropropagation. These results indicate that eradication of viruses from the old cultivars which are highly infected with several viruses is very difficult. Probably the viruses occur at very high concentrations, thereby strongly reducing the formation of new shoots and the probability to obtain new virus-free buds or shoots is therefore very low. It is noteworthy that shoot formation in tulip involves adventitious regeneration (Podwyszyńska and Marasek 2003). In such type of organogenesis, there is a vascular connection, so it is difficult to remove viruses. The shoot meristem is located very close to the mother tissue, so the mechanism of running of the new cells before viruses is extremely limited however not impossible as was shown in our study for several new cultivars and breeding lines of tulip. Similar results concerning virus eradication from bulbous species using *in vitro* techniques were also reported for shallot (Fletcher and Fletcher 1998) and lily (Blom-Barnhoorn and Aartrijk 1985). In our previous study, potyvirus-free narcissus plants were obtained from naturally infected bulbs using *in vitro* propagation via adventitious shoot regeneration without addition any antiviral compounds (Sochacki and Orlikowska 2005). Later on, we noticed difficulties with NMV eradication. NMV, of which the transmission way is unknown, was eliminated with success only in four cases among the 60 plantlets tested (Sochacki 2011) and without any success in the next two similar experiments using ribavirin. Virus-negative plants were obtained, however, in the case of aphids-transmitted NLV, a virus that is relatively easy to eliminate. It confirms the findings of Brunt *et al*. (1984) in which the elimination of potyviruses is simpler in comparison to viruses transmitted by nematodes.

In some cases of our experiments, ribavirin did not work. The reason is not clear, particularly because the exact mode of action of ribavirin against plant viruses is not well understood. On the other hand, it is known that ribavirin

reducing the growth rate of the explants, what can intensify inhibition effect in the case of old cultivars, highly infected by viruses and having low regeneration capacity. In some cases of narcissus tissues the time of exposure to ribavirin should be probably longer, what can allow to work it stronger.

Our results, both those with tulips and narcissus, confirm that antiviral compounds like ribavirin or DHT can be successfully used for virus eradication from different hosts (Spiegel and Loebenstein 1995). Similarly as in our experiments, ribavirin appeared to be effective in virus eliminating also in *Cymbidium* (Toussaint *et al.* 1994), shallot (Fletcher and Fletcher 1998), potato (Nascimento *et al.* 2003) and the previous research with narcissus (Phillips 1990; Sochacki 2011). Research done by Nascimento *et al.* (2003) on *Potato virus Y* elimination show that ribavirin was the most effective among three antiviral compounds used (ribavirin, 5-azacytidine, and 3-deazauridine). Similar, maximum effect (37% *Indian citrus ringspot virus* elimination) was seen for ribavirin followed by 2-thiouracyl and acycloguanosine. Other tested antiviral compounds – azidothymidine and DHT could not eliminate ICRSV (Sharma *et al.* 2007). A lot of research with new antiviral compounds are still leading – as experiments done by Spak *et al.* (2010) with acyclic nucleoside phosphonates – but ribavirin seems to be the most widely and the most active chemical against plant virus replication. Combination of thermotherapy and chemotherapy applied in *in vitro* conditions is commonly used for virus eradication in many ornamental pot plants and woody plants including fruit trees (Spiegel *et al.* 1993; Cieślińska 2003). However, application of thermotherapy for tulip or narcissus seems to be rather impossible. Tulip shoots cultured *in vitro* are very sensitive to temperature higher than 20°C. Culturing at higher temperature cause dormancy development tulip cultures, inhibits the regenerating ability and new shoots stop to develop (Podwyszyńska and Rojek 2008).

Our results lead to the conclusion that the process of micropropagation via adventitious shoot regeneration allowed to eliminate of viruses from infected tulips and narcissus in the case of some viruses, particularly if the concentration of virus particles in the plants is not too high. It is also essential to keep in mind that due to a possible low virus level at the *in vitro* culture stage (below the threshold of ELISA), further indexing (in consecutive propagation subcultures *in vitro* and then *ex vitro*) is imperative to confirm that the micropropagated plants are really virus-free. Problems associated with virus detection in *in vitro* growing plants were also reported by Spiegel (2006). She postulated to pay more attention to designing protocols for sampling (including tested organ and term of sampling).

Based on the results obtained in these studies, the procedure of production of tulip and narcissus virus-free stock plants was recently developed (Podwyszyńska and Sochacki 2010; Sochacki 2011).

ACKNOWLEDGEMENTS

This study was funded in part by the Polish Ministry of Scientific Research and Information Technology, project PBZ MIN-007/ P04/2003 and in part by The National Centre for Research and Development, Poland, project N R12 0063 06/2009.

REFERENCES

Anonymous (2002) EPPO Standards – Production of healthy plants for planting. Certification schemes for carnation, lily, narcissus, chrysanthemum, begonia, New Guinea hybrids of impatiens, rose, kalanchoe. *Bulletin OEPP/EPPO* **Vol. 32**, No 1, 55-66; 79-89; 91-104; 105-114; 135-145; 147-157; 159-177; 199-210

Asjes CJ (1996) Control situation of virus diseases in narcissus in the Netherlands. *Acta Horticulturae* **432**, 166-174

Asjes CJ, Brunt MH, Van Slogteren DHM (1974) Production of hyacinth mosaic virus-free hyacinth and lily symptomless virus-free lilies by meristem tip culture. *Acta Horticulturae* **36**, 223-228

Blom-Barnhoorn GJ, Van Aartrijk J (1985) The regeneration of LSV and TBV from infected *Lilium* bulb-scale explants in the presence of virasole. *Acta Horticulturae* **164**, 163-168

Brunt AA (1980) A review of problems and progress in research on viruses and virus diseases of narcissus in Britain. *Acta Horticulturae* **110**, 23-30

Brunt AA (1985) The production of virus-tested ornamental bulb crops in England; principles, practice and prognosis. *Acta Horticulturae* **164**, 153-161

Brunt AA (1995) Bulb and corm crops. *Narcissus*. In: Loebenstein G, Lawson RH, Brunt AA (Eds) *Virus and Virus-like Diseases of Bulb and Flower Crops*, John Wiley & Sons, Chichester, New York, Brisbone, Toronto, Singapore, pp 322-334

Cieślińska M (2003) Methods of eliminating viruses and viruslike pathogens from fruit trees and small fruit in *in vitro* cultures. *Postępy Nauk Rolniczych* **6**, 21-32 (in Polish)

Clark MF, Adams AN (1977) Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. *Journal of General Virology* **34**, 475-483

Clark MF, Lister RM, Bar-Joseph M (1988) ELISA techniques. In: Weissbaum A, Weissbaum H (Eds) *Methods for Plant Molecular Biology*, Academic Press, pp 507-531

Derks AFLM (1992) Some unusual serological reactions among potyviruses. *Archives of Virology* **5 (Suppl.)**, 77-79

Fletcher PJ, Fletcher DJ (1998) *In vitro* elimination of onion yellow dwarf and shallot latent viruses in shallots (*Allium cepa* var. *ascalonicum* L.). *New Zealand Journal of Crop and Horticultural Science* **26**, 23-26

Hakkaart FA, Versluijs JMA (1985) Viruses of Alstroemeria and preliminary results of meristem culture. *Acta Horticulturae* **164**, 71-75

Hanks GR (1993) *Narcissus*. In: De Hertogh AA, Le Nard M (Eds) *The Physiology of Flower Bulbs*, Elsevier, Amsterdam, The Netherlands, pp 463-558

Hansen J, Lane WD (1985) Elimination of apple chlorotic leaf spot virus from apple shoot culture by Rybavirin. *Plant Disease* **69**, 134-135

Jordan R (1989) Mapping of potyvirus-specific and group-common antigenic determinants with monoclonal antibodies by Western-blot analysis and coat protein amino acid sequence comparisons. *Phytopathology* **79**, 1157

Jordan R, Hammond J (1986) Analysis of antigenic specificity of monoclonal antibodies to several potyviruses. *Phytopathology* **76 (10)**, 1091

Kaminska M, Śliwa H, Malinowski T (2005) Partial characterisation of cucumber mosaic virus isolate infecting *Lonicera caprifolium* L. plants. *Acta Scientiarum Polonorum, Hortorum Cultus* **4 (2)**, 3-10

Lilien-Kipnis H, Stein A, Levy S (1992) The course of bean yellow mosaic virus elimination from *Gladiolus* by meristem tip culture. *Acta Horticulturae* **325**, 709-714

Mangal M, Bhardwaj SV, Handa A, Jindal KK (2003) Production of virus tested gladioli through *in vitro* and *in vivo* technique. *Acta Horticulturae* **624**, 511-514

Mowat WP (1995) Bulb and corm crops. Tulip. In: Loebenstein G, Lawson RH, Brunt AA (Eds) *Virus and Virus-like Diseases of Bulb and Flower Crops*, John Wiley & Sons, Chichester, New York, Brisbone, Toronto, Singapore, pp 352-383

Nascimento LC, Pio-Ribeiro G, Willadino L, Andrade GP (2003) Stock indexing and *Potato virus Y* elimination from potato plants cultivated *in vitro*. *Scientia Agricola* **60 (3)**, 525-530

Philips S (1990) The efficacy of four antiviral compounds in the elimination of Narcissus viruses during meristem tip culture. *Acta Horticulturae* **226**, 351-538

Podwyszyńska M, Marasek A (2003) Effect of thidiazuron and paclobutrazol on regeneration potential of tulip flower stalk explants *in vitro* and subsequent shoot multiplication. *Acta Societatis Botanicorum Poloniae* **72**, 181-190

Podwyszyńska M, Rojek A (2008) Effect of low temperature and GA_3 treatments on *in vitro* shoot multiplication of tulip (*Tulipa gesneriana* L.). *Advances of Agricultural Sciences Problem Issues* **524**, 107-113

Podwyszyńska M, Sochacki D (2010) Micropropagation of tulip: production of virus-free stock plants. In: Jain SM, Ochatt SJ (Eds) *Protocols for In Vitro Propagation of Ornamental Plants. Series: Methods in Molecular Biology*, Humana Press Inc., USA. Vol 589, pp 243-256

Podwyszyńska M, Sochacki D, Kamińska M (2005) Application of tulip *in vitro* culture for propagation of virus-free plants. *Advance of Agricultural Sciences Problem Issues* **504**, 667-674 (in Polish with English abstract and captions)

Sharma S, Singh B, Rani G, Zaidi AA, Hallan V, Nagpal A, Virk GS (2007) Production of Indian citrus ringspot virus free plants of kinnow employing chemotherapy coupled with whoot tip grafting. *Journal of Central European Agriculture* **8 (1)**, 1-8

Sochacki D (2011) The use of ELISA in the micropropagation of virus-free *Narcissus*. *Acta Horticulturae* **886**, 253-258

Sochacki D, Orlikowska T (2005) The obtaining of virus plants free from potyviruses via adventitious shoot regeneration *in vitro* from infected bulbs. *Scientia Horticulturae* **103**, 219-225

Sochacki D, Podwyszyńska M (2006) Detection of *Tulip breaking virus* (TBV) in tulips by ELISA technique. *Advance of Agricultural Sciences Problem Issues* **510**, 593-599 (in Polish with English abstract and captions)

Spak J, Holy A, Pavingerova D, Votruba I, Spakova V, Petrzik K (2010) New *in vitro* method for evaluating antiviral activity of acyclic nucleoside

phosphonates against plant viruses. *Antiviral Research* **88 (3)**, 296-303

Spiegel S, Frison EA, Converse RH (1993) Recent developments in therapy and virus-detection procedures for international movement of clonal plant germplasm. *Plant Diseases* **12**, 1176-1180

Spiegel S (2006) Problems associated with *in vitro* culture propagation and virus detection. *Acta Horticulturae* **722**, 79-82

Spiegel S, Loebestein G (1995) Control of virus diseases. In: Loebenstein G, Lawson RH, Brunt AA (Eds) *Virus and Virus-like Diseases of Bulb and Flower Crops*, John Willey & Sons, Chichester, pp 203-218

Toussaint A, Kummert J, Maroquin C, Lebrun A, Roggemans J (1993) Use of VIRAZOLE® to eradicate Odontoglossum ringspot virus from *in vitro* cultures of *Cymbidium* Sw. *Plant Cell, Tissue and Organ Culture* **32**, 303-309

Van der Linde PCG, Hol GMGM, Blom-Barnhoorn GJ, Van Aartrijk J, De Klerk GJ (1988) *In vitro* propagation of *Iris hollandica* Tub. cv. Prof. Blaauw. I. Regeneration on bulb-scale explants. *Acta Horticulturae* **226**, 121-128