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Detection and elimination of plant viruses in *Dahlia*

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Detection and elimination of plant viruses in *Dahlia*

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1. Introduction, Knowledge, Objectives

Dahlia variabilis is a very popular and economically relevant ornamental crop. Worldwide, *Dahlia* is traded as a potted or garden plant. For these purposes it is propagated vegetatively by cuttings and the division of tubers. This bears the risk of virus transmission and distribution. Virus infections in *Dahlia* are a big concern among breeders and propagators, because they decrease its economic value. One of the most important virus diseases in *Dahlia* is caused by the infection with the *Dahlia Mosaic Virus* (DMV), a Caulimovirus from the family *Caulimoviridae*. Besides mosaic patterns, DMV causes symptoms like vein clearing, shortened internodes or malformation of leaves (Pappu 2004). It is transmitted by 16 different aphids, by pollen and mechanical inoculation (Pahalawatta et al. 2007b). Latent infection and vegetative propagation of *Dahlia* are significant sources of worldwide virus distribution (Pahalawatta et al. 2007a). Other viruses are reported to infect *Dahlia* such as the two RNA-viruses, *Tobacco Streak Virus* (TSV, Ilarvirus, family *Bromoviridae*) and *Tomato Spotted Wilt Virus* (TSWV, Tospovirus, family *Bunyaviridae*), which were included in this study. The aims of this study were (i) to establish protocols for PCR-based virus detection and (ii) to test whether virus-free plants can be obtained through meristem-tip culture from 22 genotypes (historic dahlias and breeding material) that showed virus symptoms.

2. Material and Methods

In vitro culture

For 22 genotypes of *Dahlia variabilis* (Tab. 1), which showed unspecific symptoms of virus infection, meristem-tip explants were prepared. The shoot tips were surface disinfected with 70 % ethanol for 30 s, 5 % NaOCl for 15 min, followed by washing the tips in sterile water for 1, 3 and 5 min (Wang et al. 1988). The explants were incubated for six weeks on MS (Murashige and Skoog 1962) medium with 2.2 µM BAP (benzylaminopurin) and 1.1 µM IAA (indole acetic acid) at 24 °C and 16 h photoperiod(30 µmol m^{-2} s^{-1}, PAR). For shoot multiplication they were transferred to MS-medium supplemented with 0.9 µM BAP, 1.1 µM IAA and 2.9 µM GA_3 (gibberellic acid 3) and subcultured every six weeks. Rooting was obtained after two weeks on medium with 2.7 µM NAA (naphthalene acetic acid) and 2 g l^{-1} charcoal, before the plantlets were acclimatized to greenhouse conditions.

Table 1: In vitro response of 22 *Dahlia* genotypes

	Cultivar	Year	Breeder	Establishment[1]		Propagation[2]		Rooting[3]		Acclimatization[4]	
				%	Number of explants	Rate	Number of shoots	%	Number of shoots	%	Number of shoots
1	Annika		Otto/D Lüneburg	11	18	2.2	13	73.3	15	80.0	5
2	Herold		Otto/D Lüneburg	28	18	3.0	51	74.2	66	87.5	38
3	Hotzenplotz		Otto/D Lüneburg	17	12	4.6	21	42.9	35	90.0	20
4	Mikato		Otto/D Lüneburg	42	12	2.5	46	61.1	36	81.7	27
5	Optimist		Otto/D Lüneburg	42	12	3.0	49	61.3	31	95.0	18
6	H 89 M		Otto/D Lüneburg	33	12	2.9	12	20.0	5	40.0	5
7	Z 29 M		Otto/D Lüneburg	50	12	2.4	46	52.8	36	85.3	21
8	H 44 A		Otto/D Lüneburg	31	16	3.2	56	96.7	30	80.0	25
9	Sommerlachen	1944	Max Schade/D Bad Köstritz	8	12	5.0	1	-	-	-	-
10	Sieckmanns Feuerball	1934	Max Schade/D Bad Köstritz	44	16	3.1	30	28.0	25	100.0	12
11	Severins Triumpf	1932	Severin/D	50	12	3.6	38	83.3	30	86.7	30
12	Alexander von Humboldt	1960	Ernst Severin/D Berlin	0	5	-	-	-	-	-	-
13	Stolz von Berlin	1884	A. Schwiglewski/D	50	12	2.8	24	85.3	34	100.0	9
14	Kaiser Wilhelm I	1881	Chr. Deegen/D Köstritz	75	4	2.2	4	-	-	-	-
15	Prinzessin Irene von Preußen	1912	Ansorge/D	25	12	2.9	30	31.1	45	85.0	16
16	Purpurkönig	1927	Nonne & Hoepker/D	42	12	2.2	50	75.0	20	100.0	20
17	White Aster	1879	Dobbie d. Ä./GB	50	12	4.0	34	21.1	57	88.8	38
18	Ballegos Glory	1932	Ballego/NL	47	15	2.3	60	22.0	50	96.7	29
19	Panzers Rotball		Panzer	25	12	3.1	35	62.9	35	100.0	20
20	Schneeflocke	1924	Adolf Deegen/D Köstritz	42	12	3.5	28	16.1	56	96.7	28
21	Bishop of Llandaff	1928	Treseder/GB	22	18	3.1	46	34.7	49	100.0	17
22	Deuil du Roi Albert	1936	Defraignet-Troquay/FR	25	12	2.7	39	76.1	46	100.0	23

[1] percentage of meristem-tip explants which developed shoots after 6 weeks
[2] ratio of the number of shoots obtained after 6 weeks divided by the initial number of shoots (given in column)
[3] percentage of shoots which developed roots after 2 weeks
[4] percentage of shoots which survived acclimatization (data taken after 10 days)

Detection of viruses by PCR

For detection of DMV by PCR, total DNA of all genotypes was isolated from leaf material using the Nucleospin Plant II kit (Macherey & Nagel). A degenerated primer pair designed by Pahalawatta et al. (2007a) was used to detect three different strains of DMV in one PCR reaction. The PCR reaction consisted of 5 µl 10x Williams (Williams et al. 1990) buffer, 3 µl of each primer (5 pM), 4 µl dNTPs (1 mM), 0.2 µl Firepol®Taq (5 U µl-1), 3 µl

total DNA (10 ng μl^{-1}) and 9.3 µl sterile water. The reaction was carried out with 49 cycles of 30 s at 94 °C, 30 s at 39 °C and 45 s at 72 °C.

To detect TSV and TSWV, total RNA from leaf samples of 21 genotypes was extracted using the InviTrap® Spin Plant RNA Mini Kit (Stratec molecular) including a DNase treatment (14.5 µl total RNA, 2 µl 10x buffer with $MgCl_2$, 0.25 µl DNase (1 U μl^{-1}) 0.5 µl RiboLock (40 U μl^{-1}) and 4 µl sterile water) for 30 min at 37 °C and stopped by adding 1 µl EDTA (50 mM) and incubation for another 10 min. cDNA was synthesized by using primer pairs specific for the different viral sequences (TSV: 5'-CCGCTTTAACAACAACAGCA-3' and 5'-CCCCTCGACTATGGTCTTGA-3'; TSWV: 5'-GCATACTCTTTCCCCTTCTTCA -3' and 5'-AGGCAAAGACCTTGAGTTTGAG-3'). The reaction included 1 µl RNA (200-550 ng μl^{-1}) and 1 µl of the specific antisense-primer (10 pmol μl^{-1}) and was incubated for 3 min at 99 °C. After adding 2 µl 5x reaction buffer, 0.5 µl RiboLock (40 U μl^{-1}, Thermo Fisher Scientific), 0.5 µl dNTPs (10 mM), 1 µl RevertAid (2 U μl^{-1}, Reverse Transkriptase, Thermo Fisher Scientific) and 4 µl sterile water the mixture was incubated for 60 min at 42 °C. The PCR reaction consisted of 10 µl Phusion Flash Master Mix (Thermo Fisher Scientific), 1 µl of each primer (10 pM), 1 µl cDNA and 7 µl sterile water and comprised 34 cycles of 5 s at 98 °C, 5 s at 58 °C and 10 s at 72 °C. The PCR products were analyzed after electrophoresis on 1 % agarose gels. For verification of the amplified fragments the bands of the expected size were sequenced by the SeqLab company and compared to the published sequences using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).
Propagation rates of the different genotypes were tested for significance with a variance analysis (ANOVA) using the program R. For rooting, acclimatization and elimination rates no statistical analyses were carried out, because these percentage data were based on low total numbers.

3. Results

Meristem-tip cultures and in vitro propagation

The establishment of the meristem-tips was successful for 21 of the 22 genotypes (Tab. 1). The successful establishment differed from 8 % ('Sommerlachen') to 75 % ('Kaiser Wilhelm'). In total about one third survived the first six weeks after preparation, while 64 % of the explants died during this first culture phase. Contaminations were observed only in four genotypes and only in 3 % of all prepared meristem-tips (data not shown). Successful establishment was positively correlated with diameter of meristem-tips showing an optimal diameter of the explants of 1-1.2 mm (Fig. 1). Between the genotypes there was no significant difference observed for the propagation rate (ANOVA; $p = 0.855$) and most genotypes had propagation rates of about 3 (Tab. 1). In contrast, rooting ability was very different for the genotypes ranging from 16.1 % up to 96.7 %. On average, acclimatization of the plantlets to greenhouse conditions was successful for 91 % of the shoots, and for six genotypes the percentage was 100 % (Tab. 1).

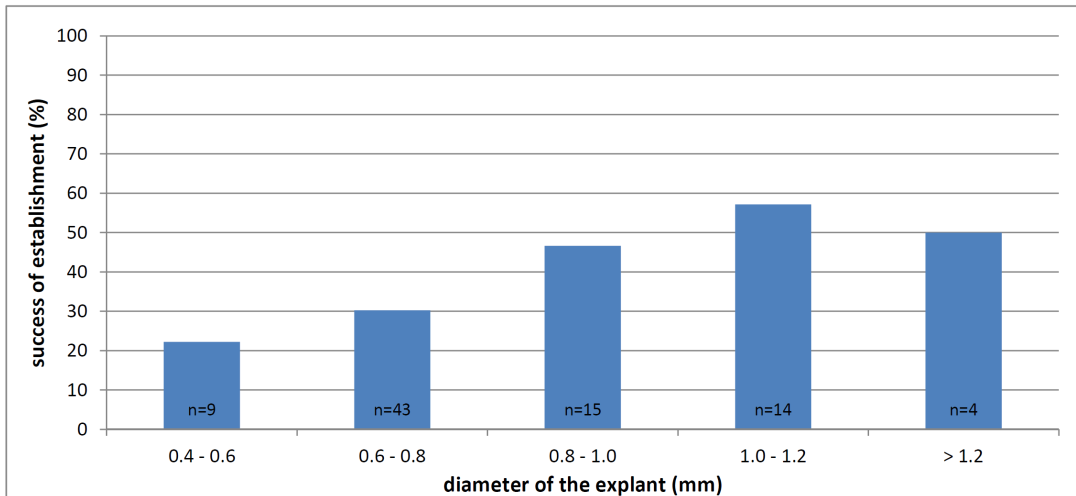

Figure 1: Successfully established cultures [%] from meristem-tip explants depending on their diameter.

Virus detection by PCR

DMV was detected in all donor plants of the 22 genotypes. Also, all acclimatized plants derived from meristem-tip culture were tested positive for DMV.

Detection of TSWV was negative for all *Dahlia* explant donor plants, although positive controls (infected *Nicotiana benthamiana* plants) showed the expected bands.

Infection with TSV in the donor plants was proven in 17 out of 21 genotypes. After meristem-tip culture 50 in vitro plantlets were again tested for this virus. TSV was not detected in 14 of them. Overall, for 9 of the 17 previously TSV infected genotypes TSV-free shoot cultures are now available.

4. Discussion

Establishing *Dahlia* in vitro by meristem-tip culture was laborious, but successful for most of the genotypes. If explants of about 0.8 mm in diameter were prepared, about half of them resulted in vigorously growing shoot cultures (Fig. 1). Although some explant donor plants were old and weak, fast growth in vitro and propagation by axillary shoot formation was observed. On the other hand, rooting strongly depended on the genotype. These results are in good agreement with those reported by Wang et al. (1988).

Elimination of DMV in *Dahlia* by meristem-tip culture was not possible. Similar results were shown by Mullin and Schlegel (1978) who first tested *Dahlia* from meristem-tip culture by means of indicator plants negative to DMV, but after ten months some plants developed symptoms again. Eid et al. (2010) showed that both in wild *Dahlia* species from Mexico and in cultivated dahlias viral sequences, which are associated with DMV, were integrated in the *Dahlia* genome. This observation gave rise to the hypothesis of a co-existence and co-evolution of pararetroviruses and their hosts (Eid et al. 2010).

The absence of TSWV may be explained by the absence of one of its main vectors *Frankliniella occidentalis* in the field. In general, the incidence of this vector is higher in the greenhouse than in the field and an infection of *Dahlia* with TSWV would be expected mainly if part of the culture would take place in the greenhouse. Similarly, Pappu et al. (2008) was not able to detect TSWV in dahlias during his study in New Zealand. In

contrast, TSWV is reported to be one of the most frequently detected viruses in *Dahlia* in the US (www.agdia.com).
The detection of TSV in *Dahlia* was also reported by Pappu et al. (2008) who tested 2 out of 11 cultivars positive for TSV by means of ELISA and RT-PCR. Transmitted by mechanical inoculation due to careless splitting of bulbs without disinfection of knifes could be an explanation for the high incidence of TSV infection in the stock of *Dahlia* tested in this study. In contrast to DMV, TSV was successfully eliminated from *Dahlia* by means of meristem-tip culture. At least part of the cells of the meristem seemed to be free of these virus particles and enabled the generation of TSV-free dahlias.

5. Conclusions

Usage of meristem-tip culture for virus elimination in *D. variabilis* does not enable the production of totally virus free plants because of the integrated sequences of a strain of DMV. Nevertheless maintenance of (historically) important cultivars is possible with this method. The developed diagnostic detection methods for TSWV and TSV enabled a quick and specific testing of plant material. Due to this procedure infections with these viruses in *Dahlia* can be identified and their distribution reduced. Further investigations may include testing of other viruses infecting *Dahlia* like *Cucumber Mosaic Virus* (CMV) as well as the specific detection of different strains of DMV.

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