Neth. J. Pl. Path. 85(1979) 39–46

Virus eradication from some Pelargonium zonale cultivars by meristem-tip culture

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Accepted 28 August 1978

Abstract

On the older leaves of *Pelargonium zonale* chlorotic rings and flecks are common, especially during spring and fall. From such plants an isometric virus can be isolated that causes local lesions on the leaves of *Chenopodium quinoa*. An attempt was made to produce symptomless plants, reacting negatively on *C. quinoa*. Meristem tips on a basal medium supplemented with α-naphthalene acetic acid and coconut milk produced abundant callus, but no plantlets. However, on media containing low concentrations of indole acetic acid and kinetin or benzyl adenine, some plants were produced that fulfilled the above requirements. Those selected on horticultural properties, are considered as valuable mother plants. Of twenty-one cultivars such plants were obtained. They may prove an important contribution to the improvement of the *P. zonale* industry in the Netherlands.

Additional keywords: Meristem-tip culture, *Pelargonium zonale*.

Introduction

Several authors have studied meristem-tip culture of *Pelargonium zonale* with various aims. One objective was the elimination of the bacterial pathogen *Xanthomonas pelargonii* (Hamdorf, 1976; Beauchesne et al., 1977; Theiler, 1977). Another purpose was vegetative propagation (Reuther, 1975; Debergh and Maene, 1977). The third objective was virus elimination (Gippert and Schmelzer, 1973; Stone et al., 1974; Hamdorf, 1976; Horst et al., 1976; Beauchesne et al., 1977; Debergh and Maene, 1977).

A survey of viruses occurring in *P. zonale* in the Netherlands was published by Van Hoof et al. (1973). Incidentally they found cucumber mosaic, tobacco necrosis, Arabis mosaic, tomato black ring and tomato ringspot viruses. Another isometric virus, however, was very common in *P. zonale*. It gave local lesions on *C. quinoa* one or two weeks after inoculation. The objective of our work, started in 1974, was to eliminate this virus.

On the older leaves of *P. zonale* chlorotic rings and flecks often occur, especially in spring and fall. The relationship between these symptoms and the commonly occurring isometric virus has not been proven, but seems likely. Therefore criteria for desired plants were absence of chlorotic rings and flecks throughout the year, and negative readings on *C. quinoa* even after repeated testing.

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Materials and methods

Pretreatment of the plant material
Before excising the meristem tips actively growing young plants were subjected to heat treatment in a glasshouse section where the temperature was gradually increased to 35 °C. A day/night interval of a few degrees prolonged plant life. Because cultivars differed considerably in heat tolerance it was not always possible to complete the desired three weeks' heat treatment.

Excising the meristem tips
After removal of the leaves the stems were superficially disinfected with bleaching liquor (5% calcium hypochlorite) for 15 minutes and then washed three times with sterile water. Both terminal and axillary meristem tips were taken, using a binocular microscope at low magnification. The tips consisted of the meristem with one or two leaf primordia (0.2–1.0 mm). They were placed on agar media in glass tubes of 2.5 × 15 mm, closed with polycarbonate caps.

Culture media
Preliminary experiments with the cultivation of meristem tips were done with α-naphthalene acetic acid (NAA) and coconut milk using a 3/4 strength Murashige and Skoog medium and Heller's micronutrients. The experiments with indole acetic acid (IAA) as an auxin were performed using Murashige and Skoog medium full strength and Heller's micronutrients. The latter medium was considered as the basal medium and modifications were in the concentrations of NH_4NO_3, FeNa-EDTA, KNO_3, $Ca(NO_3)_2 \cdot 4H_2O$, $MgSO_4 \cdot 7H_2O$ and KH_2PO_4. When an optimum of one chemical was found, study of the next chemical was performed at this optimum. For rooting the same medium was used as for growth, except that 0.5 mg/l IAA was used as an auxin and no cytokinin was added. In some experiments the effects of gibberellic acid (GA), kinetin (K), and benzyl adenine (BA) were studied. The pH of the media was adjusted to 5.7–5.9 and the tubes were autoclaved for 20 minutes at 112 °C.

Cultural conditions
The tubes were placed in a growth chamber at 19 ± 2 °C with 16 hours light of fluorescent tubes colour 33 at 5000 lx.

Transplanting
The explants were cultivated for about one month and then transferred to fresh medium of the same composition. If, after four to six weeks, sufficient growth had occurred, explants were transferred to a third tube for root formation. When some roots had developed the plantlet was transplanted into a small pot with sterilized soil which for the 24 hours was covered with a plastic lid to maintain high air humidity. Young plants were gradually hardened off until growth under normal glasshouse conditions was sufficient for sampling material for virus testing.

Virus testing
Per plant three leaves were immersed in a 1/30 *M* KNa phosphate buffer solution pH 7, supplemented with 4% polyethyleneglycol and pressed in an electric Pollähne press.

The sap was collected in plastic caps filled with 0.25 ml of the same buffer solution and rubbed onto Carborundum-dusted leaves of a *C. quinoa* plant. If after three weeks under suitable conditions no local lesions had developed, the plant was subjected to a second and, if negative, later to a third virus test. Plants that passed three tests, were considered to be free from the virus to be eliminated.

Results

Preliminary experiments with NAA and coconut milk

Some preliminary experiments were performed to obtain plants in the way as described by Pillai and Hildebrandt (1969). These authors obtained geranium callus on synthetic media with and without coconut milk and if callus produced on these media was subcultured immediately on Murashige and Skoog medium with 0.1 mg/l NAA and 10

Table 1. Effect of mineral nutrition on the growth of *Pelargonium* cultures in media with 0.1 mg/l IAA, 0.5 mg/l K and 1 mg/l GA.

Cultivar	Number of cultures	Concentration	Best concentration by visual observation
		NH_4NO_3 in mg/l	
Rubin	48	0, 206, 412, 825, 1650	206
Stadt Bern	48	0, 206, 412, 825, 1650	412
		KNO_3 in mg/l	
Jean Billes	70	2000, 3000, 4000	4000
Mercure	70	2000, 3000, 4000	4000
Mrs. F. Block	50	2000, 3000, 4000	3000
Rubin	70	2000, 3000, 4000	4000
Stadt Bern	50	2000, 2500, 3000, 3500, 4000, 4500	3500
		$Ca(NO_3)_2 \cdot 4H_2O$ replacing $CaCl_2 \cdot 2H_2O$ in mg/l	
Jardin des Plantes	49	700, 1400, 2100	700
Jean Billes	49	700, 1400, 2100	700
Mercure	49	700, 1400, 2100	700
Mrs. F. Block	49	700, 1400, 2100	700
		$MgSO_4 \cdot 7HO$ in mg/l 370, 740 combined with concentration of KH_2PO_4 in mg/l 170, 340, 510	
Beatrix	98	Six combinations	370, 340
Westfalen	98	Six combinations	370, 340
		FeNA-EDTA in mg/l	
Rubin	90	54, 108, 162, 216	54
Jardin des Plantes	49	10.8, 21.6, 32.4, 43.2, 54.0	21.6
Jean Billes	49	10.8, 21.6, 32.4, 43.2, 54.0	43.2
Mercure	49	10.8, 21.6, 32.4, 43.2, 54.0	21.6
Mrs. F. Block	49	10.8, 21.6, 32.4, 43.2, 54.0	10.8

Tabel 1. Effect van minerale voeding op de groei van Pelargonium meristeemcultures in media met 0,1 mg/l IAA, 0,5 mg/l K en 1 mg/l GA.

mg/l K and incubated at 16/8 h light/dark cycle, shoots were induced in 8–10 weeks and roots in another 8°10 weeks. Therefore in 1974 several tests with NAA and coconut milk were performed. From twelve cultivars 48 explants per medium were placed on Murashige and Skoog medium 3/4 strength supplemented with 2.5 mg/l, 6 mg/l and 10 mg/l NAA, respectively, all with 2.5 mg/l K and 150 ml/l coconut milk. The cultivars were Cherry Blossom, Genie Irene, Improved Ricard, Jardin des Plantes, Jean Billes, Lerchenmüller, Maloja, Mercure, Mrs. F. Block, Rubin, Snowmass and Stadt Bern. A big yellowish-white callus usually formed that after a few months turned brownish and died. Transplanting at monthly intervals kept the calluses alive, but hardly any differentiation into shoots or roots took place.

With the cultivars Genie Irene and Jean Billes meristem tips were first cultured on a 3/4 strength Murashige and Skoog medium supplemented with 2.5 mg/l NAA and 150 ml/l coconut milk. The calluses obtained were subcultured on a medium with 0.1 mg/l NAA and 10 mg/l K, also very similar to that described by Pillai and Hildebrandt (1969). Although abundant callus formation took place, differentiation was not achieved. Since this approach yielded no plants, research with NAA and coconut milk was abandoned.

Effect of mineral nutrition on the growth in vitro

In 1975 a series of experiments were performed with as basal medium Murashige and Skoog full strength, microelements after Heller and a growth substance composition of 0.1 mg/l IAA, 0.5 mg/l K and 1 mg/l GA. The isolated meristem tips usually formed a small compact callus of a few mm at the basis of the explant. This type of callus differed from the loose brittle type obtained with NAA in the former experiments. A few leaves and a small stem were formed and transplanting after a month favoured further growth. Many cultures, however, turned yellow before root development and died. Therefore studies were done with some mineral elements to improve growth. As shown in Table 1, all elements studied affected growth, but the optimum concentration differed with the cultivar. Apparently the yellowish colour of many cultures was not caused by a lack of nitrogen or iron. Replacing $CaCl_2 \cdot 2H_2O$ by $Ca(NO_3)_2 \cdot 4H_2O$ to avoid a possibly toxic effect of the chloride ion did not solve this problem either.

From the tests the following composition resulted as useful, although not optimal.

Table 2. Number of plants obtained by meristem tip culture on media with 0.1 mg/l IAA, 0.5 mg/l K and 1 mg/l GA, followed by 0.5 mg/l IAA and no K. Compilation of several experiments.

Cultivar	Number of cultures	Number of plants obtained in soil	Number of plants with no reaction on *C. quinoa*
Beatrix	588	68	26 (4.4%)
Jardin des Plantes	147	4	2 (1.3%)
Jean Billes	442	35	26 (5.8%)
Mercure	247	15	11 (4.4%)
Rubin	352	11	7 (1.9%)
Stadt Bern	346	56	11 (3.1%)
Westfalen	588	21	8 (1.3%)

Tabel 2. Aantallen door meristeemcultuur verkregen planten gekweekt op bodems met 0,1 mg/l IAA, 0,5 mg/l K en 1 mg/l GA, gevolgd door 0,5 mg/l IAA en geen K. Samenvoeging van meerdere proeven per ras.

Table 3. Effect of kinetin concentration at a level of 0.1 mg/l IAA and 1 mg/l GA.

Cultivar	Number of cultures	Kinetin concentration in mg/l	Number of plants obtained in soil	Number of plants with no reaction on *C. quinoa*
Jean Billes	97	0.5	26	21
Jean Billes	97	1	24	19
Jean Billes	97	5	0	0
Jean Billes	97	10	0	0
Lerchenmüller	98	0.5	3	3
Lerchenmüller	98	1	3	3
Lerchenmüller	98	5	0	0
Lerchenmüller	98	10	0	0
Rubin	90	0.5	5	5
Rubin	90	1	20	16
Rubin	90	5	1	1
Rubin	90	10	0	0

Tabel 3. Effect van kinetine concentratie bij een niveau van 0,1 mg/l IAA en 1 mg/l GA.

NH_4NO_3	400 mg/l	$MgSO_4 \cdot 7H_2O$	370 mg/l
KNO_3	4000 mg/l	KH_2PO_4	340 mg/l
$Ca(NO_3)_2 \cdot 4H_2O$	700 mg/l	FeNa-EDTA	40 mg/l

When during these experiments cultures were obtained of about 1 cm they were transferred to a rooting medium with 0.5 mg/l IAA and no K to produce plantlets transferable to soil. The ultimate yield of plants is presented in Table 2. The percentage of negatively reacting plants varied with the cultivar but was generally low.

Effect of kinetin concentration and of benzyl adenine

Three cultivars were subjected to a range of K concentrations (Table 3). Both 0.5 mg/l and 1 mg/l were useful, whereas 5 mg/l and 10 mg/l proved toxic. With two other cultivars K and BA were compared at the 0.5 mg/l and 1 mg/l level (Table 4). With both substances few plants were obtained.

Table 4. Comparison of kinetin (K) and benzyl adenine (BA) at a level of 0.1 mg/l IAA and 1 mg/l GA.

Cultivar	Number of cultures	Cytokinin concentration in mg/l	Number of plants obtained in soil	Number of plants with no reaction on *C. quinoa*
Mrs. F. Block	98	0.5 K	1	1
Mrs. F. Block	98	0.5 BA	2	2
Mrs. F. Block	98	1 K	9	8
Mrs. F. Block	98	1 BA	2	2
Verbeterde Rubin	98	0.5 K	1	1
Verbeterde Rubin	98	0.5 BA	4	2
Verbeterde Rubin	98	1 K	3	3
Verbeterde Rubin	98	1 BA	4	3

Tabel 4. Vergelijking van kinetine met benzyl adenine bij een niveau van 0,1 mg/l IAA en 1 mg/l GA.

Horticultural aspects

The plants tested on *C. quinoa* and apparently free from the isometric virus did not show the chlorotic symptoms on the older leaves throughout the year. Propagation of these plants may be a method to control this virus, provided quick reinfection of nuclear stock plants can be avoided. Unfortunately the vector(s) of this virus is/are not yet known. Before the plants are to be propagated, however, a stringent selection on horticultural properties is essential. Therefore, from fall of 1975 onwards, plants were made available to some growers for evaluation and selection. Until middle of 1978 twenty-one cultivars, freed from the isometric virus, have been under trial, selection and propagation. From several thousands of these plants an impression was obtained about the horticultural performance. As was expected, the effect of virus eradication varied between cultivars, but several improvements were noticed. Flowering was more abundant, plants sometimes produced more flower stalks, or formed more florets per flower head. Vegetative growth was not excessive, but of the desired more compact type. There was less root rot in the cuttings struck during the winter months. Cutting production of the mother plants had, at least in some cultivars (e.g. Dark Red Irene), increased. An impression of the reinfection of the nuclear stock plants was obtained in 1978 by testing 280 plants (eleven cvs). Seven plants (2.5%) contained virus and were destroyed. The horticultural performance combined with the acceptable reinfection rate warrant further trials and more general use of virus-tested plants obtained as described above.

Discussion

On media with NAA and coconut milk callus formation was easy but differentiation into shoots and roots very difficult, and results, as described by Pillai and Hildebrandt (1969), were not obtained. This cannot be attributed to the use of different cultivars alone, because we used cv. Genie, as did these authors. A concentration of 4000 mg/l KNO_3 in the medium may seem high. However, in studies with the cv. Stadt Bern, Hamdorf (1976) also found that high levels of nitrate, in this case NH_4NO_3, were desirable for shoot growth.

The toxicity of kinetin concentrations of 5 mg/l and 10 mg/l was also reported by Gippert and Schmelzer (1973) and Reuther (1975). Therefore the observation of Debergh and Maene (1977) is interesting: they avoided the toxic effect of the high kinetin concentration by keeping the tips first on a medium without growth substances for one week and then transplanting them to a medium with the high kinetin concentration.

Theiler (1977) used BA as a cytokinin in a 0.2 mg/l concentration. We obtained plants on media containing 0.5 mg/l and 1 mg/l of BA and it may be concluded that several cytokinins, such as kinetin and BA, are useful and that no specific substance is required.

During this study no attempt was made to achieve vegetative propagation in vitro, nor were specific tests performed to demonstrate the absence of *Xanthomonas pelargonii* in the plants produced. An isometric virus was eliminated. Since we tested our plants on *C. quinoa* other viruses that react on this indicator species may also be claimed to be absent, e.g. Arabis mosaic virus. The performance of several thousands of plants obtained after propagation from plants produced via our procedure has been

encouraging. More profuse flowering of plants of a desired compact habit, less root rot of the cuttings during the winter months and a higher cutting production with some cultivars combined with an acceptably low rate of reinfection, are advantages achieved. It is concluded that wider use of this plant material may improve the quality of *P. zonale* growing in the Netherlands.

Samenvatting

Viruseliminering uit enkele Pelargonium zonale cultivars door meristeemcultuur

In *P. zonale* komt veelvuldig een ziektebeeld voor, gekenmerkt door chlorotische vlekken en kringen in de oudere bladeren, vooral waarneembaar in het voor- en najaar. Uit dergelijke planten kan een isometrisch virus worden geïsoleerd, dat lokale lesies veroorzaakt op *C. quinoa*. In dit onderzoek werd getracht door middel van meristeemcultuur plantmateriaal te produceren, dat zowel symptoomloos is gedurende het gehele jaar, alsook negatief reageert in de toets op *C. quinoa*. Explantaten op voedingsbodems met NAA en cocosnootmelk produceerden veel callus, maar leverden geen plantjes op. Meer succes werd bereikt met lage concentraties van IAA en kinetine (Tabel 2). Onderzoek over de optimale samenstelling van de voedingsbodem met betrekking tot stikstof, ijzer, calcium, magnesium en fosfaat werd uitgevoerd (Tabel 1). Ook met benzyladenine werden planten verkregen, die symptoomloos bleken en bovendien negatief reageerden op *C. quinoa* (Tabel 4). Alvorens in aanmerking te komen voor vermeerdering werden de verkregen planten grondig op tuinbouwkundige eigenschappen geselecteerd. Na deze selectie bleek het aan de praktijk beschikbaar gestelde materiaal rijker te bloeien met behoud van een compacte groeiwijze. Er trad minder uitval door wortelrot op bij het stekken in de wintermaanden en ook was de stekproduktie van de moederplanten soms hoger. Ofschoon de vector van het virus nog niet bekend is en bescherming van het gezonde materiaal tegen herinfectie dus niet gericht kan plaatsvinden, bleef de herbesmetting toch aanvaardbaar laag (2,5% in 1978). We concluderen dat algemeen gebruik van het op boven beschreven wijze verkregen plantmateriaal een belangrijke bijdrage kan betekenen tot het verhogen van het kwaliteitsniveau in de Nederlandse *P. zonale* teelt.

Acknowledgments

We like to thank the General Netherlands Inspection Service (N.A.K.S.) for the use of their growth chamber, the firm A. Verhoog, Maasdijk, for the supply of plant material and Mr F. de Jonge for his skilful assistance.

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