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Hemerocallis hybrid 'Revolute' Establishment of virus-free plants, in vitro propagation and storage

Hemerocallis-*hybrid 'Revolute'* *Etablering af virusfrie planter, in vitro-formering og opbevaring*

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Summary

The *Hermerocallis* hybrid 'Revolute' was recently selected as one of the very best cultivars of the daylilies.

Unfortunately, this cultivar was found to be infected with arabis mosaic virus.

As the aim was to improve the plant material, it was decided to clean the plants for existing viruses and furthermore to develop a rapid in vitro propagation method.

The identification of the arabis mosaic virus infection was based on indicator plants and *i*mmuno *s*orbent *e*lectron *m*icroscopy (ISEM).

Virus-free *Hemerocallis* plants were established by meristems excised from plants which had been heat-treated at 30 to 34°C for two to seven months.

A tissue culture of *Hemerocallis* was achieved with the *Murashige* and *Skoog* 1962 (MS-62) medium in 50 p.c. concentration.

Shoots, roots and callus were established by adding phytohormones comprising different cytokinins and auxins.

The weight of the callus production was more than doubled every month.

The regeneration of adventitious shoots from callus cultures was achieved during a period of four to six months, in which the regeneration rate was increased.

The number of regenerated shoots per tube during three generations were 9.5, 10.0 and 4.5, respectively.

Plants in vitro were successfully stored during half a year at 12°C.

Key words: *Hemerocallis,* arabis mosaic virus, in vitro propagation, virus-free plants, in vitro storage.

Resumé

Hemerocallis-hybriden 'Revolute' er for nylig blevet selekteret som en af de bedste sorter af daglilje.

Ved rutinetest af plantematerialet viste det sig, at denne sort uheldigvis var inficeret med arabismosaikvirus.

Da formålet var at forbedre plantematerialet, blev det besluttet dels at rense sorten for eksisterende virus, dels at udvikle en hurtig in vitro opformeringsmetode.

Identifikationen af det påviste arabismosaikvirus blev baseret på indikatorplanter og immuno sorbent electronmikroskopi (ISEM).

Virusfri *Hemerocallis*-planter er blevet etableret fra meristemer skåret udelukkende fra varmebehandlede planter dyrket ved 30 til 34°C gennem to til syv måneder.

Vævskultur af *Hemerocallis* blev etableret ved anvendelse af *Murashige* og *Skoogs* medium fra 1962 (MS-62) i halv styrke.

Skud, rod og kallus blev udviklet ved tilsætning af forskellige cytokininer og auxiner.

Kallusproduktionen målt i mg blev mere end fordoblet hver måned.

Adventive skud blev udviklet fra kalluskulturer i løbet af fire til seks måneder med en stigende regenerationsrate.

Antal regenererede skud pr. glas gennem tre generationer, blev opgjort til henholdsvis 9,5, 10,0 og 4,5.

Planter i rørglas er med et godt resultat blevet opbevaret i et halvt år ved 12°C.

Nøgleord: *Hemerocallis,* arabismosaikvirus, in vitro formering, virusfrie planter, in vitro opbevaring.

Introduction

In order to improve the plant material of daylily *(Hemerocallis)* selection work has been carried out comprising a collection of different cultivars and clones.

From selected cultivars plant material was further tested for possible existing viruses.

One of the most promising cultivars 'Revolute' unfortunately proved to be infected by arabis mosaic virus (6). Even if the infection did not cause appreciable symptoms in leaves and flowers, it was decided to try to establish virus-free plants in order to achieve healthy nuclear stock plants.

No other virus infection has earlier been described in *Hemerocallis.*

As propagation of *Hemerocallis* in the traditional way by division is very slow, it was furthermore suggested to develop an in vitro propagation method for rapid propagation purposes. In vitro plantlet formation and organogenesis in daylily callus have been described earlier (1,2).

The experiments concerning these aims were carried out during the years 1984 to 1988, and the results are described in this paper.

Method

The plant material consisted of a selected clone of the cultivar 'Revolute' received from the Institute of Landscape Plants, Hornum.

The infected plants were grown partly under normal glasshouse conditions (20°C during the day and 18°C during the night) and partly in a thermostatically regulated growth chamber either at 30 or 34 ± 1°C for 16 hours followed by a night temperature of 20°C for 8 hours. Irradiation 3.6 W/m PAR was provided by Philips 30 W/33 flourescent lamp.

Bio-assay

Leaves from the *Hemerocallis* hybrid were tested for virus infection to the indicator plant *Chenopodium quinoa.* The virus was further transferred to other indicator plants for diagnostic purposes.

As transmission method, sap inoculation was used comprising carborundum powder (400 mesh) and a potassium/phosphate buffer pH 7.6 including 4 p.c. polyethylene glycol (MW 6000).

The established meristem plants were all tested on *Chenopodium quinoa.* The results obtained were estimated according to the development of systemic chlorosis which indicated the presence of a virus infection (4).

Immuno sorbent electron microscopy was used to identify the virus. The procedure was based on the method described by *Milne* and *Luisoni* (3) and modified by adding 2 p.c. polyethylene glycol (MW 6000) in 0.1 m phosphate buffer pH 7.

Sap from virus infected indicator plants was used as antigen.

Antiserum against the arabis mosaic virus originated from *E. P. Valence,* Hungary.

Tissue culture

A meristem culture and a callus culture were both started from apical meristems, which were excised from untreated or heat-treated plants after two to seven months of treatment.

The size of the meristems was 0.25 mm including 1–2 pairs of leaf primordia.

All the meristems from *Hemerocallis* are situated at the very base of the plant, which means beneath the soil surface. This causes a very high rate of contaminations and difficulties despite the use of desinfectants.

Searching for possible lateral meristems at the floral stems gave a negative result.

To reduce the contamination risk, the soil surface down to the root region was top dressed with inorganic granulated, non-absorbent stone wool (blue Grodan).

As desinfectant, a 3 p.c. solution of Korsolin was used during 15 min. The treated plant material was allowed to dry up before use.

The meristem culture was started and established in MS-62 medium (5) using either 100 or 50 p.c. of the macro elements.

As growth regulators, 1 mg/liter of furfuryl amino purine (FAP) and 0.2 mg/liter of indolyl butyric acid (IBA) were used.

As rooting medium, 50 p.c. MS-62 with addition of 0.1 mg/l IBA was used.

To stimulate the callus production, the 50 p.c. MS-62 medium was used with the following modifications (M-MS-62, 50 p.c.): KNO_3 1250 mg/l, KH_2PO_4 125 mg/l, biotin 0.05 mg/l, folic acid 0.5 mg/l and with the omission of casein hydrolysate. As growth regulators, 0.2 mg/l IBA, 0.5 mg/l indolyl acetic acid (IAA), 0.1-3.0 mg/l 6 (γ, γ - dimethylallylamino)-purine (2-ip) and 0.1 mg/l benzyl amino purine (BAP) were used.

The growth regulators were all added under sterile conditions after the autoclaving.

The tissue cultures were subcultured every month or every other month on the same medium. After establishment of growth, the meristem plant or callus tissue were transferred to other media to obtain shoot and root formation.

The rate of the callus production was measured by weighing the produced callus every month. After this the callus was devided into two parts and transferred to a medium for further callus growth and for shoot production, respectively. The number of developed shoots were also counted.

Plantlets ready for potting were achieved after about 6–8 months of culture in a growth room at 20°C day/18°C night and 16 hours' photoperiod. The irradiance was 10 Wm^{-2} PAR provided by Philips TLF 40 W/33 cool white fluorescent lamp.

Preliminary experiments concerning storage of tissue culture in vitro at low temperatures was carried out.

Established plants grown in medium 50 p.c. MS-62 with addition of 0.1 mg/l IBA were stored during half a year in a temperature regulated growth room, at 12°C ±1°C and with 16 hours' photoperiod.

Results

Symptomatology

Viruslike symptoms consisting of chlorotic to white streaks along the veins have been observed once in older leaves from virus infected plants grown in greenhouse during the month of April (Fig. 1).

Bio-assay

Virus was transmitted directly from symptomless *Hemerocallis* leaves to *Chenopodium quinoa* by sap inoculation, causing chlorotic mottle and deformation in the new growth.

The virus was transferred to other indicator plants, causing the same symptoms in *Chenopodium amaranticolor,* local lesions and systemic mosaic followed by growth decline in *Cucumis sativus* and red spots in *Phaseolus vulgaris.*

No visible symptoms were observed in *Nicotiana clevelandii, N. tabacum* 'Samsun' and 'Xanthi'.

ISEM

Sap from systemic infected cucumber plants was used as antigen for the ISEM-test comprising the following antisera: Arabis mosaic virus, cucumber mosaic virus, tobacco ringspot virus, tomato black ring virus and tomato ringspot virus.

The virus reacted only with antiserum to arabis mosaic virus with a complete decoration of the virus particles.

Fig. 1. Leaves from *Hemerocallis* infected with arabis mosaic virus.
Blade af Hemerocallis *inficeret med arabismosiakvirus.*

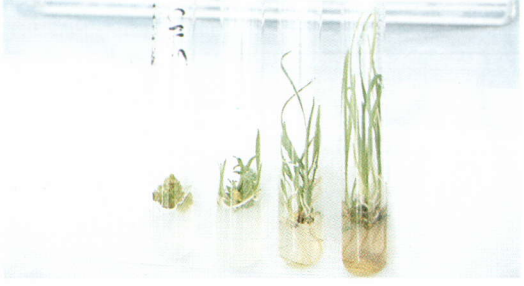

Fig. 2. (From left to right): 1. Callus growth after 12 days, and 2. callus and shoot regeneration after 4.5 months both in the modified medium M-MS62, 50 p.c. with addition of 0.1 mg/l BAP, 0.1 mg/l 2-ip and 0.5 mg/l IAA. 3. Shoot development 2.5 months after transfer of callus with shoot initials, and 4. *Hemerocallis* plant ready to be potted 1 month after transfer of shoot both to the medium MS-62, 50 p.c. with addition of 0.1 mg/l IBA.
(Fra venstre til højre): 1. Kallusvækst efter 12 dage, og 2. Kallus med skudvækst efter 4,5 måneder i det modificerede medium M-MS-62, 50 pct. suppleret med 0,1 mg/l BAP, 0,1 mg/l 2-ip og 0,5 mg/l IAA. 3. Skududvikling 2,5 måneder efter flytning af kallus med skudinitialer, og 4. Hemerocallis *plante klar til potning 1 måned efter flytning af skud til mediet MS-62, 50 pct. suppleret med 0,1 mg/l IBA.*

Fig. 3. Healthy meristem plants established in soil.
Sunde meristemplanter etableret i jord.

Photos: J. Begtrup, N. Paludan.

Establishment of virus-free plants

Established meristem plants from untreated and heat treated plants were tested to *Chenopodium quinoa* for existing arabis mosaic virus. The results are shown in Table 1.

Table 1. Inactivation of arabis mosaic virus in *Hemerocallis* using heat treatment and meristem culture.
Inaktivering af arabismosaikvirus i Hemerocallis ved varmebehandling og meristemkultur.

Heat treatment *Varmebehandling*		Meristem plants *Meristemplanter*	
Months *måneder*	°C	Total No. *antal i alt*	Virus-free p.c. *pct.*
0	20	29	0
2	30	4	75
7	30	11	100
2.5	34	31	100

Adventitious shoots from untreated virus infected meristem plants were furthermore established and tested for AMV. Of totally 22 adventitious plants three were found virus-free (14 p.c.).

Virus-free plants originating from apical meristems were delivered to the Institute of Landscape Plants as a basic material for future nuclear stock plants.

Furthermore, 100 virus-free and 100 virus infected *Hemerocallis* plants all originating from adventitious shoots were also delivered in order to control the genetical stability (true to type) of the plant material.

Tissue culture

The establishment of a tissue culture from *Hemerocallis* is shown in Table 2.

Normal shoot production was achieved using the phytohormones FAP and IBA and the development of roots on medium containing IBA only (Fig. 2).

The plants were ready to be potted three to six months after excision of the meristems, depending on the preceding treatment of the material. The heat treatment delayed the date of potting.

The length of the leaves were measured at the time of potting, from 60 to 80 mm, and the root development estimated as optimal (Fig. 2).

Callus growth was mainly induced in a medium containing the phytohormone 2-ip, comprising 40 p.c. of the cultures (Table 2).

Further callus production followed by shoot regeneration was achieved using the modified M-MS62, 50 p.c. medium supplemented with 0.1 mg/l 2-ip, 0.1 mg/l BAP and 0.5 mg/l IAA. Further shoot development was achieved with MS-62, 50 p.c. added only 0.1 mg/l IBA (Fig. 2).

The results are shown in Table 3 and 4.

The callus production measured in mg has been more or less doubled up every month, and the propagation rate has been high during all three generations.

However, only a certain percentage of the tubes with callus are inducing shoots as shown in Table 4.

The shoot regeneration has been nearly the same during the first and the second generation, whereas the third generation developed only half the amount of shoots. However, the results from the third generation were evaluated after 4 months, and not five and six months after, which has influenced the result. Also, the third generation comprised fewer tubes with shoot regeneration (Table 4).

Storage

Storage of established plants in vitro at 12°C during half a year has proved possible. During storage new green leaves were developed, whereas the original leaves gradually became whitish. Of totally 37 stored plants 35 could be further grown on or potted directly in soil.

Table 2. The establishment of a tissue culture of *Hemerocallis* comprising apical meristem plants, potted plants and development of callus.
Etablering af vævskultur af Hemerocallis *som apikale meristemplanter, pottede planter og udvikling af kallus.*

Plant Material Treatment *Plantemateriale Behandling*	Media *Medium*	Concen-[1] tration *Koncentration* p.c./*pct.*	Phytohormones *Vækststoffer* mg/liter	Excised total *Skåret antal*	Established *Etableret* p.c./*pct.*	Potted plants *Pottede planter* p.c./*pct.*	Callus growth *Kallusvækst* p.c./*pct.*
None	MS-62	50	1 FAP 0.2 IBA	32	94	60	10
2–7 mo at 30°C	– –	50	– –	18	100	89	6
2,5 mo – 34°C	– –	100	– –	57	84	71	2[2]
None	– –	50	3 2-ip –	25	76	16	40[3]

1) Macro elements only
Kun makronæringsstoffer
2) Virus-free callus used for in vitro propagation
Virusfri kallus brugt til in vitro-formering
3) Virus infected callus used for in vitro propagation
Virusinficeret kallus brugt til in vitro-formering.

Table 3. Callus and shoot production from *Hemerocallis* tissue during three generations.
Kallus- og skudproduktion fra Hemerocallis *væv gennem tre generationer.*

Subject/*emne*	Generations/*generationer*		
	1	2	3
Callus production/*kallusproduktion*			
mg callus introduced in 3 × 48 tubes *mg kallus placeret i 3 × 48 glas*	8070	6960	6960
mg callus harvested after 1 month *mg kallus høstet efter 1 måned*	15700	16650	15950
mg callus produced during 1 month *mg kallus produceret efter 1 måned*	7630	9690	8990
mg callus per tube in average during 1 month *mg kallus pr. glas i gns. efter 1 måned*	159.0	201.9	187.3
Multiplication rate/month *Formeringsfaktor/måned*	1.95	2.39	2.29
Shoot production/*skudproduktion*			
mg callus introduced in 3 × 48 tubes *mg kallus placeret i 3 × 48 glas*	5170	7480	9200
No. of regenerated shoots 6, 5 and 4 months later *Antal regenererede skud 6,5 og 4 måneder senere*	484	482	218
No. of shoots per tube, average of all tubes *Antal skud pr. glas i gns. af alle glas*	9.5	10.0	4.5
mg callus per shoot, average *mg kallus per skud i gns.*	10.7	15.5	42.2

Table 4. Adventitious shoots regenerated from *Hemerocallis* tissue during three generations.
Adventive skud regenereret fra Hemerocallis *væv gennem tre generationer.*

Generation *Generation*	Percentage of tubes[1] with shoot regeneration during months *Procent rørglas[1] med skudregenerering efter antal måneder*					
	1	2	3	4	5	6
1	24	45	53	–	–	53
2	42	44	–	–	63	–
3	25	–	–	42	–	–

1) Total of 3 × 48 tubes
I alt 3 × 48 glas

Discussion

The arabis mosaic virus infection of *Hemerocallis* is the first report of a virus attack on plants of this genus.

The effect of this virus infection still has to be investigated, but during the present work, weak leaf symptoms have only sporadically occurred. The erradication of the arabis mosaic virus was only achieved by using meristems from heat-treated plant material. The same result has been found for other nepo viruses like the tomato ringspot virus in *Pentas lanceolata* (*Paludan,* not published), and the tomato black ring virus in *Arabis* (7). Only heat treatment at 34°C was effective in both cases.

A tissue culture of *Hemerocallis* has earlier been described comprising organogenesis and regeneration of plants from callus originating from different organs as petals, floral stalks and roots (1,2).

The callus developed from the meristematic tissue in this experiment is similar to the described flower petal callus (2), i.e. compact, yellowish-green in colour and with green initials.

Induction of callus has mainly been achieved with 2-ip and further callus growth with a combi-

nation of 2-ip, BAP and IAA. Regeneration of adventitious shoots has occurred on this medium also, during a period of several months, whereas new growth of callus was induced by a transfer to fresh medium.

This is in contrast to earlier experiments (1,2), where a callus production was achieved in liquid or solid medium containing (2.4 dichlorophenoxy) acetic acid (2.4-D) and shoots when transferred to a medium without 2.4-D.

The present results demonstrate that whole daylily plants can be regenerated from meristematic callus tissue. Even if the established plants look identical with the parent plants, it is important to investigate the genetical stability of the plants originating from the shoots. This work will be carried out during 1989 comprising virus infected and virus-free plants.

Conclusion

Arabis mosaic virus was diagnosed in *Hemerocallis* 'Revolute' showing temporary chlorotic to white streaks in the leaves during the spring.

The diagnosis was based on indicator plants and ISEM.

Virus-free *Hemerocallis* plants were established from meristems excised from heat-treated plants only. The temperatures 30° or 34°C during two months or more were both effective.

A tissue culture of *Hemerocallis* was easily achieved with the MS-62 medium used in a 50 p.c. concentration. Single shoots were developed by adding the phytohormones FAP (1 mg/l) and IBA (0.2 mg/l), and roots by adding (0.1 mg/l) IBA only.

Callus and regenerated adventitious shoots were produced on medium M-MS-62, 50 p.c. added 2-ip (0.1 mg/l), BAP (0.1 mg/l) and IAA (0.5 mg/l).

The callus weight was more than doubled every month as an average of three generations, the multiplication rate being 1.95, 2.39 and 2.29, respectively.

The regeneration of adventitious shoots occurred approximately in 50 p.c. of the callus cultures with an increase in the percentage during the first three to five months.

The number of regenerated shoots per tube achieved during three generations, where the results were calculated after six, five and four months of cultivation, was on an average 9.5, 10.0 and 4.5, respectively.

Established plants in vitro were successfully stored during half a year at 12°C.

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