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# Inactivation of poinsettia mosaic virus and poinsettia cryptic virus in *Euphorbia pulcherrima* using heat treated mini-cuttings and meristem-tip culture

*Inaktivering af poinsettia-mosaikvirus og poinsettia-crypticvirus i* Euphorbia pulcherrima *ved varmebehandling af ministiklinger og meristemkultur*

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**Summary**

*Euphorbia pulcherrima* plants are frequently infected with both poinsettia mosaic virus (PMV) and poinsettia cryptic virus (PCV). Infections of these viruses cause mosaic and deformations of both leaves and bracts.

To avoid these symptoms, virus free plants can be produced by the use of effective methods such as heat treatment of mini-cuttings and meristem-tip culture. Both methods are described in this paper.

Inactivation of PMV and PCV has been achieved using both methods, the PMV to a very high degree. A prolongation of the heat treatment increased the rate of inactivation for both viruses.

Submergence in a 3 per cent »Korsolin« solution for 30 to 45 minutes produced a successful disinfection of the excised mini-cuttings. The effect depended on both the variety and the duration of the treatment.

The meristem-tip culture is an easier and faster method to achieve a very high rate of virus free plants.

Normal shoot development has been achieved by both meristem-tip culture and mini-cuttings irrespective of the heat treatment performed. Deformation of shoots has been accelerated by both the use of longer disinfection periods and high salt concentration in the growing medium.

**Key words:** *Euphorbia,* poinsettia mosaic virus, poinsettia cryptic virus, heat treatment, inactivation, mini-cuttings, meristem-tip culture, immunosorbent electron microscopy, disinfection.

**Resumé**

*Euphorbia pulcherrima* er ofte inficeret med både poinsettia-mosaikvirus (PMV) og poinsettia-crypticvirus (PCV). Infektion af disse 2 virus forårsager mosaiksymptomer og deformiteter i både blade og højblade.

For at undgå disse symptomer, kan virusfrie planter produceres ved effektive metoder som f.eks. varmebehandling af ministiklinger og meristemkultur, der begge er beskrevet i denne beretning.

Inaktivering af PMV og PCV er opnået ved begge metoder. PMV var lettere at inaktivere end PCV. Inaktiveringen øgedes for begge virus ved en forlængelse af varmebehandlingsperioden.

Desinfektionen af ministiklingerne var effektiv ved anvendelse af en 3% »Korsolin« opløsning i 30 til 45 minutter. Effekten var afhængig af både sorten og behandlingens længde.

Meristemkulturer er en lettere og hurtigere metode til at opnå en høj procentdel virusfri planter.

En normal skududvikling er opnået både med ministiklinger og meristemkultur uafhængig af den udførte varmebehandling. Dannelsen af deforme skud er imidlertid øget med henholdsvis en længere desinfektionstid og en højere saltkoncentration i næringsmediet.

**Nøgleord:** *Euphorbia,* poinsettia-mosaikvirus, poinsettia-crypticvirus, varmebehandling, inaktivering, ministiklinger, meristemkultur, immunoelektronmikroskopi, desinfektion.

## Introduction

Mosaic and deformed leaves and bracts in *Euphorbia pulcherrima* are caused by virus infection. The virus is described and diagnosed as a tymo-like virus named poinsettia mosaic virus (3,4,5,6).

Poinsettia mosaic virus is commonly found in *Euphorbia pulcherrima* both in North America and in several countries in Europe (2,6).

Another spherical virus called poinsettia cryptic virus was found in *Euphorbia pulcherrima* and described as a latent seedborn virus (5).

In Denmark the 2 viruses mentioned were found in *Euphorbia pulcherrima* in 1980 and also in *E. fulgens* in 1984 (8,11) (fig. 1).

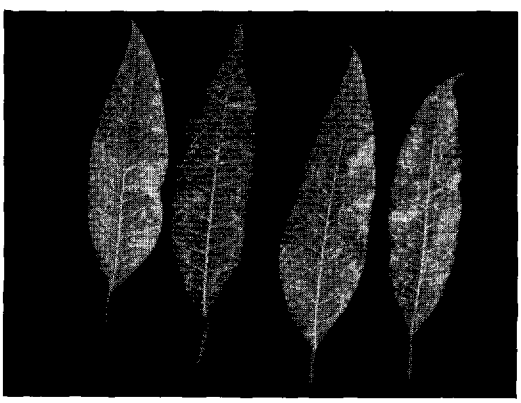

Fig. 1. *Euphorbia fulgens* with clear mosaic symptoms in the leaves. Poinsettia mosaic virus and poinsettia cryptic virus have both been shown.
*Euphorbia fulgens* med kraftig mosaik i bladene, hvor både poinsettia-mosaikvirus og poinsettia-crypticvirus er påvist.

Experiments to establish virus free plants have been carried out with success using different methods such as heat treatment of minicuttings, cell suspension culture and meristem-tip culture (9,10,12,13).

Investigations, carried out at the Institute of Plant Pathology in Lyngby in 1982, comprising 22 different varieties of poinsettia showed that all the tested plants were infected with poinsettia mosaic virus (PMV) and/or with poinsettia cryptic virus (PCV), regardless of existent symptoms.

This result showed a need for a method by which virus free plants could be established. This paper deals with the possibilities of producing virus free plants by the use of heat treatment of mini-cuttings, which was tested during 1983 and 1984, and meristem-tip culture in 1985.

## Method

*Plant material and growth conditions*

The material consisted of the following poinsettia varieties: 'Dark Annette', 'Snowstar', 'Femina' and 'WSE 8000' all received from the Institute of Glasshouse Crops, Årslev.

All the plants were infected with both PMV and PCV, based on immuno electron microscopy tests (ISEM).

The plants were grown under normal greenhouse conditions at 20°C during the day and 18°C during the night. The plants were illuminated during the winter season given long-day-treatment to avoid development of buds and flowers.

Well established plants were placed in a thermostat regulated growth chamber with a day

temperature for 16 hours of 34 ± 1°C and an illumination (Philips 30W/33 fluorescent lamp) of 3.6 W $m^2$ followed by a night temperature for 8 hours of 20°C in darkness.

*Mini-cuttings, disinfection and growing medium*
Mini-cuttings from 1 to 1.5 cm long were taken from the top of the shoots from the plants after a period of 2, 3.5, 5, 7, 8.5, 10.5, 12.5 and 14 months of heat treatment respectively.

The cuttings, with all developed leaves removed, were disinfected in a 3 per cent solution of »Korsolin« for periods varying from 5 to 45 minutes and then rinsed in 3 baths of sterilized and distilled water.

The disinfected mini-cuttings were transferred to tubes containing growth medium under sterile conditions and placed in a growth room at 20°C with 16 hours illumination (Philips flourescent lamp 'cool white' TLF 40W/33) with 10 W $m^2$. The medium used was the one described by *Preil et al.* (13) consisting of *Murashige* and *Skoog* 1962 (MS-62) added 2 ppm indolyl acetic acid (IAA), 0.2 ppm benzyl amino purin (BAP), 3 per cent saccharose and 0.6 per cent agar. The pH-value was measured before the autoclaving and adjusted to 6.

*Meristem-tip culture*
Meristem-tips were excised from *Euphorbia* plants grown in a greenhouse and supplied with water and nutrients by trickle irrigation. No disinfection was performed. The size was 0.25 mm including 1–2 leaf primordials. The MS-62 medium was used with different concentrations of the nutrient solution comprising macro and micro elements, sugar, vitamines and amino acids and with different combinations of the phytohormones benzyl amino purin (BAP) and indolyl acetic acid (IAA). The phytohormones were all added under sterile conditions after the autoclaving.

A modification of the MS-62 medium was also used comprising a 75 per cent medium concentration with a reduction of the $NH_4NO_3$ to 638 mg/l and the $KNO_3$ to 1425 mg/l omission of the casein hydrolisate and addition of L-cysteine to 2 mg/l. The phytohormones consisted of furfuryl amino purin (FAP) and indolyl butyric acid (IBA) in different concentrations.

Different pH-levels of the medium ranging from 5 to 8 have also been tried.

The meristem-tips were grown under the same conditions as mentioned for the mini-cuttings. The meristem-tips were usually transferred once to the medium originally used and then to the last mentioned modified MS-62 medium with a very low content of phytohormones or none at all in order to stimulate normal growth and root development (fig. 2).

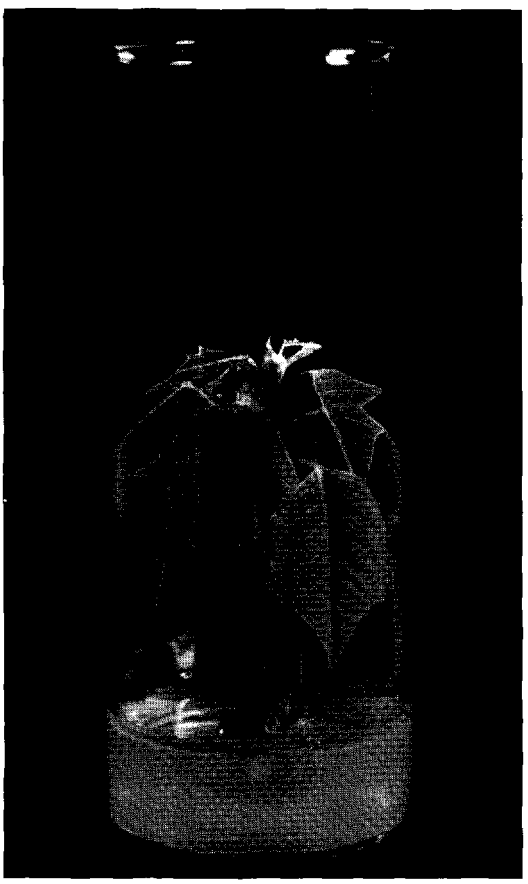

Fig. 2. *Euphorbia pulcherrima* shoot deriving from a meristem-tip and transplanted to a bigger glass jar for stimulation of growth.
*Euphorbia pulcherrima*-skud skåret som et meristem og senere flyttet til et glas for at stimulere væksten.

*Virus test*

Immunosorbent electron miscroscopy (ISEM) was used to identify the viruses poinsettia mosaic virus and poinsettia cryptic virus. The ISEM test was carried out in accordance with the original procedure (7), but 2 per cent polyethylene glycol was added to the suggested phosphate buffer (1,14) in order to solve tannin, lignin and other problems in sap from many woody plants, which generally cause difficulties when electron microscopic grids are prepared for ISEM decoration.

In this experiment sap from young leaves was used either from plants grown in greenhouse or from the heat treated mini-cuttings and meristem-tip plants, both in tubes (fig. 3). All the samples were later retested once or twice, if virus infection could not be shown by the first test.

The results from the ISEM test were based on decorated virus particles found by scanning of at least 5 squares per grid (400 mesh). The 2 antisera used were kindly supplied by *Renate Koenig*, Braunschweig.

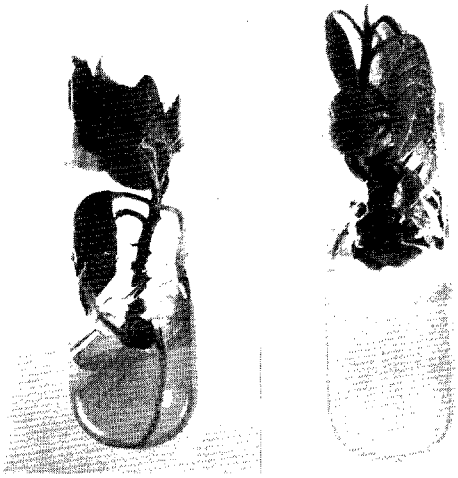

Fig. 3. *Euphorbia pulcherrima* shoots deriving from mini-cuttings with and without roots. Leaves from plants in this size were used for the ISEM-test performed.
*Euphorbia pulcherrima*-skud fra ministiklinger med og uden rødder. Blade fra planter af denne størrelse blev anvendt ved den gennemførte ISEM-test.
Fotos: Jens Begtrup.

## Results

*Disinfection of mini-cuttings*

The effect of the disinfection with »Korsolin« comprising treatments of varying duration in 3 varieties is shown in table 1.

**Table 1.** The influence of »Korsolin« treatment on the rate of contamination and development of normal shoot growth in mini-cuttings excised from heat treated mother plants.
*Indflydelsen af »Korsolin«-behandling på forureningsgraden og udviklingen af normal skudvækst hos ministiklinger taget fra varmebehandlede moderplanter.*

| Varieties | »Korsolin« treatment in minutes | Mini-cuttings in test tubes *Ministiklinger i rørglas* | | |
|---|---|---|---|---|
| | | total no. | contaminated per cent | with normal shoot growth[1] per cent |
| *Sorter* | *»Korsolin« behandling i minutter* | *antal i alt* | *forurenede %* | *med normal skudvækst[1] %* |
| 'Dark Annette' | 30 | 75 | 88 | 67 |
| | 45 | 150 | 55 | 63 |
| 'Snowstar' | 5 | 50 | 66 | 94 |
| | 30 | 204 | 23 | 82 |
| | 45 | 50 | 30 | 26 |
| 'WSE 8000' | 30 | 181 | 23 | 74 |
| | 45 | 50 | 4 | 58 |

[1] Based on tested samples from tubes which were not contaminated.
*Baseret på testede prøver, der ikke var forurenede.*

The rate of the contamination depends on both the variety and the duration of treatment. The variety 'Dark Annette' has been more difficult to disinfect compared to the other varieties, and 5 minutes of treatment is too short to reduce the contamination to an acceptable level.

The effect of the »Korsolin« treatment on the development of normal shoot growth shows that a prolonged treatment decreases the number of normal shoots.

*Heat treatment of mini-cuttings*

The influence of the heat treatment on the development of normal shoot growth is shown in table 2.

**Table 2.** The influence of heat treatment on the development of normal shoot growth in mini-cuttings as an average of 3 varieties[1].
*Varmebehandlings indflydelse på udviklingen af normal skudvækst hos ministiklinger som et gns. af 3 sorter[1].*

| Heat treatment in months at 34°C<br>*Varmebehandling i måneder ved 34°C* | Mini-cuttings in test tubes *Ministiklinger i rørglas*<br>not contaminated no.<br>*antal ikke forurenede i alt* | with normal shoot growth per cent<br>*med normal skudvækst %* |
|---|---|---|
| 0 | 75 | 81 |
| 2 | 36 | 89 |
| 3.5 | 57 | 79 |
| 5 | 64 | 95 |
| 8.5 | 71 | 44 |
| 10.5 | 69 | 90 |

[1] 'Dark Annette', 'Snowstar', 'WSE 8000'.

The heat treatment did not have any influence on the development of normal shoot growth.

*Inactivation of PMV and PCV by heat treatment of mini-cuttings*

The inactivation of the 2 viruses by the use of heat treated mini-cuttings is shown in table 3.

**Table 3.** Inactivation of PMV and PCV by heat treatment of mini-cuttings as an average of 3 varieties[1].
*Inaktivering af PMV og PCV ved varmebehandling af ministiklinger som et gns. af 3 sorter[1].*

| Heat treatment in months at 34°C<br>*Varmebehandling i måneder ved 34°* | Mini-cuttings in test tubes *Ministiklinger i rørglas*<br>total no.<br>*antal i alt* | virus free[2]<br>PMV per cent<br>% | <br>PCV per cent<br>% |
|---|---|---|---|
| 0 | 13 | 0 | 0 |
| 2 | 31 | 3 | 0 |
| 3.5 | 45 | 0 | 0 |
| 5 | 96 | 10 | 1 |
| 7 | 39 | 56 | 28 |
| 8.5 | 36 | 69 | 33 |
| 10.5 | 62 | 44 | 5 |
| 12.5 | 16 | 75 | 38 |
| 14 | 10 | 100 | 80 |

[1] 'Dark Annette', 'Snowstar', 'WSE 8000'.
[2] Based on 2 or 3 repeated negative ISEM tests.
*Baseret på 2 eller 3 gentagne negative ISEM testninger.*

The percentage of virus free plants increases with the prolonged heat treatment. The PMV is easier to eliminate than the PCV.

The virus concentration of the PMV has constantly been lower than the PCV based on the amount of particles found by the ISEM-scanning.

In addition, the results from each of the single varieties 'Dark Annette', 'Snowstar' and 'WSE 8000' as an average of 2 to 8.5 months of the heat treatment shows that the percentage of plants free from PMV was 31, 27 and 17 respectively. Correspondingly, the percentage was 10, 15, and 5 for PCV.

*Meristem-tip culture*

The influence of the different media on the establishment of meristem-tips and the development of normal shoot growth is shown in table 4.

The content of inorganic macro compounds (mg/l) in the different media is as follows: MS-62 (no. 1): 100 per cent 4530, 75 per cent 3398, 50 per cent 2265, 33 per cent 1495 and in the modified MS-medium (no. 2): 100 per cent 2799.

Concentrations from 1495–2799 mg/l gave far

**Table 4.** The influence of the used media on the establishment of meristem-tips and normal shoot growth.
*Næringsmediets indflydelse på etableringen af meristemer og normal skudvækst.*

| Varieties *Sorter* | Media[1] No. *Nr.* | Concentration *Koncentration* % | Phytohormones *Vækststoffer* mg/l BAP | IAA | pH-value *pH-værdi* | Meristem-tips Excised total *Antal skåret* | Established per cent *Etableret %* | Normal shoot growth per cent *Normal skudvækst %* |
|---|---|---|---|---|---|---|---|---|
| 'Snowstar' | 1[2] | 100 | 0.2–0.5 | 0.2–2 | 6 | 78 | 69 | 0 |
| 'Femina' | » | » | » | » | » | 78 | 72 | 4 |
| 'WSE 8000' | » | » | 0.2 | 1 | » | 25 | 100 | 8 |
| » | » | 75 | » | » | » | 25 | 92 | 4 |
| » | » | 50 | » | » | » | 50 | 80 | 58 |
| » | » | 33 | » | » | » | 25 | 72 | 44 |
| » | » | 50 | 0.5 | 0.2 | » | 25 | 84 | 48 |
| » | » | » | 0.2 | 0.2 | » | 25 | 84 | 38 |
| » | 2[3] | 100 | 0.2 | 0.2 | » | 25 | 48 | 25 |
| | | | FAP | IAA | | | | |
| » | 2 | 100 | 1 | 0.2 | 5.5 | 50 | 74 | 54 |
| | | | FAP | IBA | | | | |
| 'Dark Annette' | 2 | 100 | 1 | 0.2 | 5 | 50 | 52 | 35 |
| | » | » | » | » | 5.5 | 50 | 80 | 28 |
| 'Snowstar' | » | » | » | » | 6 | 75 | 76 | 28 |
| 'Femina' | » | » | » | » | 7 | 50 | 76 | 24 |
| | » | » | » | » | 8 | 50 | 78 | 21 |

[1] The meristem-tips were transferred once to the medium originally used and later to medium 2 with phytohormones in low concentration or completely without hormones.
*Meristemerne blev flyttet første gang til det oprindelig anvendte medium og derefter til medium 2 med vækststoffer i lav koncentration eller helt uden.*

[2] MS-62 medium in different concentrations comprising macro and micro elements, sugar, vitamines and amino acids.
*MS-62 medium i forskellige koncentrationer af makro- og mikronæringsstoffer, sukker, vitaminer og aminosyrer.*

[3] Modified MS-62 media.
*Modificerede MS-62 medier.*

the best result, while higher salt concentrations produced very few normal shoots.

The lowest pH-value at 5 was the best with a decrease in the number of normal shoot growth towards higher pH-levels using the modified MS-medium (no. 2).

The leaf colour, shape and structure together with the shoot development was closer to the normal appearence, using the FAP/IBA phytohormones compared with the BAP/IAA.

*The inactivation of PMV and PCV by meristem-tip culture*

The inactivation of the 2 viruses by meristem-tip culture in 4 varieties is shown in table 5.

The virus inactivation depends on both the virus itself and the variety as found ealier for the mini-cuttings. The rate of virus free plants is high.

*Repetition of the ISEM test*

All the virusfree (negative) samples which were found during the first tests of both the heat treated mini-cuttings and the meristem-tips were retested once or twice.

Of 130 samples free of PMV, 90 per cent were virusfree also in the second test, and 86 per cent (based on 49 samples) in the third test.

Concerning the PCV-tests the corresponding results were 81 (based on 87 samples) and 75 per cent (based on 20 samples) respectively.

**Table 5.** Inactivation of PMV and PCV by meristem-tip culture.
*Inaktivering af PMV og PCV ved meristemkultur.*

| Varieties *Sorter* | Meristem-tips *Meristemer* | | |
|---|---|---|---|
| | total no. *antal i alt* | virus free[1] PMV p.cent % | virus free[1] PCV p.cent % |
| 'Dark Annette' | 20 | 95 | 90 |
| 'Snowstar' | 20 | 85 | 60 |
| 'Femina' | 7 | 71 | 29 |
| 'WSE 8000' | 43 | 54 | 42 |
| Average *Gns.* | 90 | 71 | 56 |

[1] Based on 2 or 3 repeated negative ISEM tests.
*Baseret på 2 eller 3 gentagne negative ISEM testninger.*

Although the total number of virusfree samples decreases with the repetition of the tests, the rate of virusfree plants is high and reliable.

## Discussion

Before the meristem-tip culture was introduced and used as a method to establish virus free plants, rooting of mini-cuttings from heat treated plants was the method generally used.

Heat therapy of poinsettia mosaic (12) led to heat treatment of mini-cuttings.

The results achieved correspond with *Pfannenstiel et al.* (12) showing that it is possible to inactivate the PMV. But the present results also show that it has been more difficult and required far longer periods (7 months) of heat treatment to gain PMV-free plants, even with the use of smaller mini-cuttings (1–1.5 cm compared with 2–3 cm) and a higher temperature (34°C compared with 32°C).

The inactivation of the PCV has been successful, but constantly on a lower level.

The inactivation of the PMV and the PCV was much more effective with the meristem-tip culture, and this corresponds with the results from the paper by *Preil et al.* (13) describing the use of cell suspension culture, where both viruses were almost completely eradicated. This method can only be used with plants which give uniform regenerates (homohistonts).

In order to avoid any kind of mutations in plant material not being genetically constant (chimera) the meristem-tip culture has to be used.

The certainty of the ISEM-test is based on repeated tests of treated samples, where virus infection could not be shown at the first test.

The decrease in the total number of virusfree samples shown during the repetition is not unusual in treated material. This is normally due to a very low virus concentration difficult to diagnose followed by an increase in the virus concentration during the development of new growth, where the virus is more easily diagnosed. Therefore the repetition of the tests is most important to obtain a high level of certainty.

During the performance of the ISEM-tests some samples, especially deriving from long-term heat treated material, were difficult to analyse.

The virus particles became only more or less partially decorated, the particles probably being partly destroyed during the heat treatment.

## Conclusion

Virus free plants have been established both by heat treated mini-cuttings and by meristem-tip culture.

The inactivation rate depends on the virus itself, the variety and the duration of the heat treatment respectively. The PVC was more difficult to eliminate than the PMV. The longer the heat treatment the higher the rate of virus free plants.

The heat treatment has not had any harmful effects on the growth of the developed shoots, whereas this was the case with a long disinfection treatment using »Korsolin«. Mini-cuttings without contamination were achieved with the use of a shorter disinfection treatment.

Media with low salt concentration, low pH-value and the use of the FAP/IBA phytohormones gave the best quality of the developed shoots.

## Litterature


1. *Accotto, G. P.* 1982. Immunosorbent electron microscopy for detection of fanleaf virus in grapevine. Phytopath. medit. 21, 75–78.
2. *Chiko, A. W.* 1983. Poinsettia mosaic virus in British Columbia. Plant Disease 67, 427–428.
3. *Fulton, R. W., Worf, G. L., Gaard, G. & Heimann, Mary Francis* 1978. Detection of a virus assicated with a bract-deforming disease of poinsettia. Phytopathol. News 12, 198.
4. *Fulton, R. W. & Fulton, Jeanette L.* 1980. Characterization of a tymo-like virus common in poinsettia. Phytopathology 70, 321–324.
5. *Koenig, Renate & Lesemann, D.-E.* 1980. Two isometric viruses in poinsettias. Plant Disease 64, 782–784.
6. *Lesemann, D.-E., Koenig, R., Huth, W., Brunt, A. A., Phillips, S. & Barton, R. J.* 1983. Poinsettia Mosaic Virus – a Tymo virus? Phytopath. Z. 107, 250–262.
7. *Milne, R. G. & Luisoni, E.* 1975. Rapid high resolution immune electron microscopy of plant viruses. Virology 68, 270–274.
8. *Paludan, N. & Thomsen, A.* 1981. New attacks of virus diseases 1980. Plant diseases and pests in Denmark 1980, 97th annual report, 47.
9. *Paludan, N. & Thomsen, A.* 1983. Virus diseases of ornamental plants. Plant diseases and pests in Denmark 1982, 99th annual report, 44–45.
10. *Paludan N. & Thomsen, A.* 1984. Virus diseases of ornamental plants. Plant diseases and pests in Denmark 1983, 100th annual report, 85.
11. *Paludan, Niels* 1984. Virus i *Euphorbia.* Gartner Tidende 100, 1309.
12. *Pfannenstiel, Mary Ann, Mintz, Keith P. & Fulton, Robert W.* 1982. Evaluation of heat therapy of poinsettia mosaic and characterization of the viral components. Phytopathology 72, 252–254.
13. *Preil, W., Koenig, Renate, Engelhardt, Margarete & Meier-Dinkel, A.* 1982. Eliminierung von Poinsettia mosaic Virus und Poinsettia cryptic Virus aus *Euphorbia pulcherrima* Willd. durch Zellsuspensionskultur 1982. Phytopath. Z. 105, 193–197.
14. *Russo, M., Martelli, G. P. & Savino, V.* 1982. Immunosorbent electron microscopy for detecting sap-transmissible viruses of grapevine. Proc., Seventh Meeting, International Council for the study of Viruses and Virus-like diseases of the Grapevine: Niagara Falls, Canada, Sept. 8–12, 1980, pp. 251–257.


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