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Detection of chrysanthemum stunt and potato spindle tuber viroids by polyacrylamide gelelectrophoresis

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Abstract

Stunt viroid can be detected in chrysanthemums with the polyacrylamide gelelectrophoresis (PAGE) method developed by Morris and Smith (1977) for potato spindle tuber viroid. The time of sample preparation can even be shortened considerably. The reliability of the short and the complete PAGE method proved to be similar to that of the biological 'Mistletoe' test in a parallel experiment. Combined samples can be tested in the complete PAGE method easily permitting the detection of one diseased chrysanthemum top in a total of ten.

Although potato spindle tuber viroid is not known to occur in the Netherlands we searched for methods to detect possible infections. Artificial infections of tomato and potato plants and of sprouts of potato tubers could readily be detected by Morris and Smith's method. Using this method it was possible to demonstrate infections by severe and weak isolates even when not yet producing symptoms. In tomato plants the viroid could be detected four to eight days before symptoms appeared.

Introduction

Diseases caused by viroids are increasingly attracting attention not only because of the interesting molecular biology of viroids but also since they are highly infectious and of economic importance. In the Netherlands chrysanthemum stunt is well known (Noordam, 1952) and pale fruit disease of cucumber, more recently described in this country (Van Dorst and Peters, 1974), may be of potential importance. Potato spindle tuber has not yet been found here.

Efficient and reliable routine methods to test plant material for the presence of chrysanthemum stunt viroid (CSV) and for potato spindle tuber viroid (PSTV) are essential to further improve the quality of Dutch chrysanthemum propagation material and to protect our seed potato industry. Assaying plant material for viroids with test plants is laborious, and time- and glasshouse space-consuming because diseases caused by viroids have long incubation periods. For example, in testing chrysanthemums for stunt by grafting onto chrysanthemum 'Mistletoe' it usually takes six to eight weeks before symptoms develop, but the incubation period may well last several months. The technique of Morris and co-workers (Morris and Wright, 1975; Morris and Smith, 1977) to detect PSTV nucleic acid in infected tissue is also laborious, because it includes specific precipitation, dialysis, alcohol precipitation and polyacrylamide gelelectrophoresis (PAGE), but it only takes two to three days to be completed.

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We have tested Morris' method to see whether the PAGE method is reliable under our conditions for PSTV and CSV, and can also be used to detect latent infections. In addition we have tried to simplify the method for large-scale routine application.

Materials and methods

Viroids and plant material. CSV-infected and healthy chrysanthemums from various cultivars were obtained from the Research Station for Floriculture and from commercial growers through the Netherlands General Inspection Service for Ornamental plants. Grafts were made from naturally infected plants and the scion-stock combinations were maintained in a glasshouse at 26°C.

For research on PSTV under special safeguards a license was obtained from the Netherlands Plant Protection Service. Dr P. J. Howell (Agricultural Scientific Services, East Craigs, Scotland) provided freeze dried leaf material of a severe and a weak isolate, and Dr R. P. Singh (Agriculture Canada, Research Station, Fredericton, Canada) sent tubers with the Saco isolate. Dr T. J. Morris (University of New Brunswick, Fredericton, Canada) supplied healthy and PSTV-infected 'Kennebec' potato tubers. A tomato plant infected with a fifth isolate was obtained from Dr D. Peters (Agricultural University, Wageningen, the Netherlands). This isolate was originally obtained from Dr R. P. Singh. Only the severe Howell isolate induced severe symptoms in tomato and potato under prevailing conditions, the four others produced weak symptoms. The tubers were either induced to sprout at room temperature or potted to produce plants. The latter was done in a glasshouse between 20 and 35°C, depending on the amount of sunshine, and in natural light, or in a growth chamber at 30°C (day) and 25°C (night) and a high intensity of artificial light for 16 h a day.

'Sheyenne' and 'Rutgers' tomato plants were also grown under these conditions. They were inoculated by rubbing carborundum-dusted leaves of two to three week-old plants, then having four or five leaves, with sap from PSTV-infected potato or tomato plants. Diseased potato plants were obtained by growing them from infected tubers or by mechanical inoculation.

Extraction of viroids was according to the method of Morris and Smith (1977) modified as follows. If available, 5 g of young shoots were taken instead of 1 g and 2-mercaptoethanol was not used. The buffer-tissue ratio as described by Morris and Smith was used for potato and tomato plants, but for chrysanthemum material the amount of buffer was doubled. Extraction was accomplished by mixing for 30 seconds with an Ultraturrax homogenizer in an ice-bath. All centrifugings were done in a Sorvall low-speed centrifuge using Corex tubes. The nucleic acids extracted from 5 g of material were resuspended in 0.5 ml of water after the final ethanol precipitation.

Polyacrylamide gelelectrophoresis for nucleic acid separation was on 5% polyacrylamide gels according to Morris and Smith (1977). However, the gels were pre-run for 30 min at 3.5 mA/gel and after loading the gels, the samples were first run for 15 min at 3.5 mA/gel and then at 6 mA/gel till the bromophenol blue tracer dye was 1 cm from the bottom of the gel. Electrophoresis was performed at room temperature, without cooling, and was completed within $2\frac{1}{2}$ h. The gels were removed from the

tubes and stained overnight with 0.1% toluidine blue 0 in water. They were destained with distilled water. After several changes of the water the gels were examined over a light panel.

Results

Detection of CSV. Working with chrysanthemum tops at a buffer-tissue ratio of 1:2 the amount of supernatant after the first low-speed centrifuging was too small. When using twice the amount of buffer perfect results were obtained (Fig. 1). The number of bands may vary. In Fig. 1A and B, for example, there is a band of host nucleic acid just below the viroid band (V). The presence of this band in healthy samples and the fact that the electrophoresis front is not always at the same place, may make it hard to decide whether or not the viroid is present. Therefore the presence of the viroid was always checked by its relative mobility (R_m) of 0.61 against the 5S RNA band which is always present. To enable rapid determination of this R_m value the nomogram presented in Fig. 2 was made on a transparency over the light panel. By placing the stained gel in such a position that the meniscus is on the meniscus line and its 5S band on the base line, a viroid band at the $R_m = 0.61$ position can immediately be recognized.

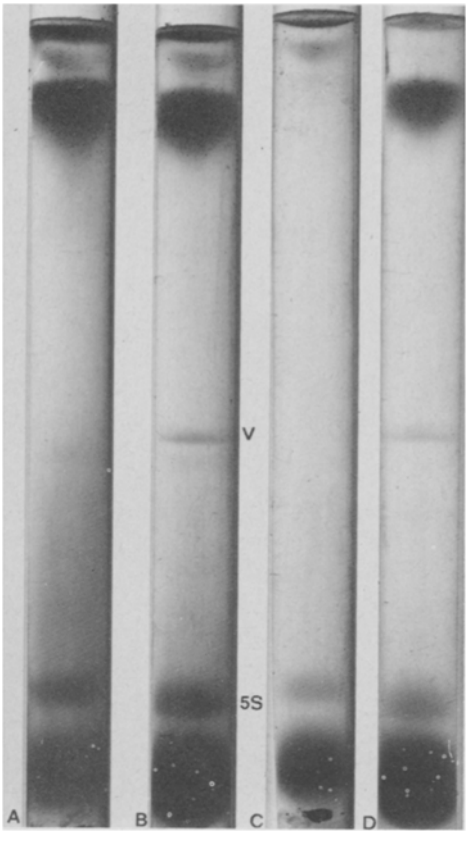

Fig. 1. Electropherograms of chrysanthemum extracts. Healthy (A) and CSV-infected (B) 'Golden Horim', healthy 'Golden Horim' (C) and CSV-infected 'Pink Marble' (D). V = viroid band.

Fig. 1. Elektroferogrammen van chrysanteëxtracten Gezonde (A) en met CSV geïnfecteerde (B) 'Golden Horim', gezonde 'Golden Horim' (C) en met CSV geïnfecteerde 'Pink Marble' (D) V = viroïdeband.

Fig. 2. Nomogram for localisation of viroid band. See text for explanation.

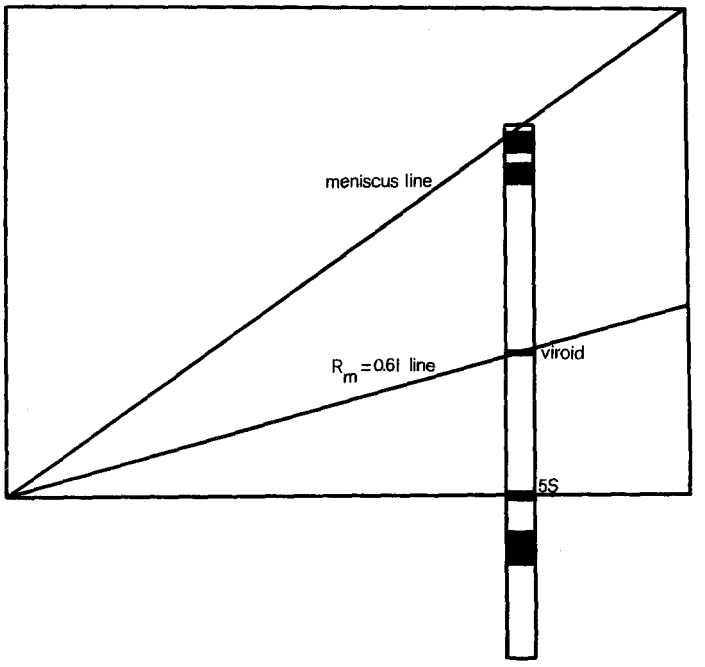

Fig. 2. Nomogram voor het vaststellen van eventuele viroïdeband in een gel. Zie tekst voor verklaring.

So far we tested infected material of the chrysanthemum cvs Bonnie Jean, Golden Horim, Judith, Milonka, Mistletoe, and Pink Marble. With all of them it was possible to detect CSV by the typical viroid band after PAGE.

In the routine procedure always frozen material was used but results with fresh material were equally good. However, frozen material is easier to homogenize.

When tested separately, leaves and stems of the tops of chrysanthemum plants proved to contain similar concentrations of viroid. However, with stems a more compact interphase was obtained after the first low-speed centrifuging as compared to leaves. In the latter case it was difficult to remove the supernatant because it was largely absorbed by the loose interphase.

Usually all steps of the method were performed at 0 to 4°C, keeping the tubes in ice and using pre-cooled rotors. However, carrying out the method, with exception of the dialysis step, at room temperature, led to equally good results.

The sensitivity of the method was compared to that of the 'Mistletoe' method in March 1977 when 50 chrysanthemum plants of the cvs Milonka, Mistletoe and Pink Marble were tested simultaneously by grafting onto 'Mistletoe' and by the PAGE method. Diseased and healthy plants, as well as plants of unknown condition were included. Plant tops were harvested and used for PAGE; on each remaining stem a healthy 'Mistletoe' top was grafted. The tests were performed by two groups of workers and those who performed the PAGE method did not know which plants were diseased. The results they produced three to four days after the start of the ex-

periment were in complete agreement with those of the grafting test that was read two months later.

To enable PAGE testing of more samples per day, it seemed necessary either to simplify the method or to combine samples.

The first simplification as compared to Morris' original method has already been mentioned. A considerable gain in time is obtained by using an Ultraturrax instead of a Virtis homogenizer because the latter is hard to clean between samples. The Ultraturrax can simply be washed by operating it in a container with running tap water.

Several other ways to produce sap were tested, such as using a power-driven Pollähne press, but they all had the disadvantage that, in order to mix the sap with the denaturating phenol mixture, another homogenizing device was needed.

In other attempts to shorten the method steps, such as dialysis, LiCl precipitation and the final ethanol precipitation, or combinations of these, were omitted. Omitting the dialysis was found to lead to a too high ionic strength of the sample. Even if the material was not homogenized in the extraction buffer, but in the less concentrated electrophoresis buffer, no viroid bands were observed after PAGE if the dialysis step had been left out.

The removal of large nucleic acids by LiCl precipitation is not necessary to obtain a good viroid band and this also holds for the concentration step. Even if both steps were omitted and the material was homogenized, centrifuged and dialyzed, it could successfully be tested for viroids with PAGE (Fig. 3). As expected, the viroid bands were not as dense as with the complete method, but still readily detectable. The gels on which samples of the short method were run showed more background in the region where the viroid band tends to appear. The amount of background seemed to depend on the chrysanthemum cultivars used. 'Mistletoe' induced far more background than 'Bonnie Jean' and 'Pink Marble'. We tried to diminish this background by using other extraction buffers, additives like 2-mercaptoethanol and diethyldithiocarbamate, using stems instead of leaves etc., but none of these modifications had a favourable effect.

The short method was tested in another parallel experiment, carried out in July 1977, in which 50 plants of the cvs Bonnie Jean, Judith, Golden Horim, Milonka, Mistletoe and Pink Marble were investigated with the 'Mistletoe' test, the short PAGE method and the complete PAGE method. For the short PAGE method 1 ml of the first supernatant was taken and dialyzed separately against water, the remainder was handled according to the complete method. The results showed complete agreement for the three tests. All the ten samples of 'Judith' and of 'Pink Marble', as well as the five samples of 'Milonka' and of 'Mistletoe' reacted positively; the ten samples of 'Bonnie Jean' all reacted negatively, while from the ten 'Golden Horim' samples two reacted positively and eight negatively.

The effect of combining samples was tested as follows. Samples of ten tops of 1 g each were made with various combinations of healthy 'Bonnie Jean' and diseased 'Pink Marble' as indicated in Table 1. The samples were tested by the short and the complete PAGE method. With the complete method one diseased top in a total of ten could easily be detected and the band was so dense that one diseased top in a total of 20 might well have been detectable. With the short method a faint band was observed with one or two diseased tops in a total of ten. However ,with cultivars causing

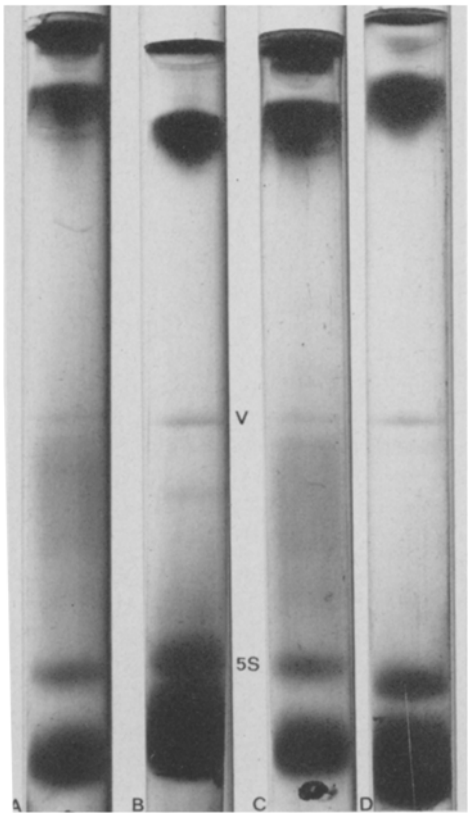

Fig. 3. Electropherograms of extracts of CSV-infected chrysanthemums. Extracts of 'Judith', prepared by the short (A) and the complete (B) method, and extracts of 'Golden Horim', prepared by the short (C) and the complete (D) method. V = viroid band.

Fig. 3. Elektroferogrammen van extracten van met CSV geïnfecteerde chrysanten. Extracten van 'Judith', bereid volgens de korte (A) en volledige (B) methode en extracten van 'Golden Horim', bereid volgens de korte (C) en de volledige (D) methode. V = viroïdeband.

Table 1. Detection of CSV in combined samples of ten tops of chrysanthemum plants, each weighing 1 g, using the short and the complete PAGE methods. – = no band; ± = faint band; + = clearly visible band.

Sample		PAGE method	
		short	complete
10 healthy 'Bonnie Jean' tops		–	–
9 healthy 'Bonnie Jean' tops +	1 diseased 'Pink Marble' top	±	+
8 healthy 'Bonnie Jean' tops +	2 diseased 'Pink Marble' tops	±	+
7 healthy 'Bonnie Jean' tops +	3 diseased 'Pink Marble' tops	+	+
6 healthy 'Bonnie Jean' tops +	4 diseased 'Pink Marble' tops	+	+
5 healthy 'Bonnie Jean' tops +	5 diseased 'Pink Marble' tops	+	+
4 healthy 'Bonnie Jean' tops +	6 diseased 'Pink Marble' tops	+	+
3 healthy 'Bonnie Jean' tops +	7 diseased 'Pink Marble' tops	+	+
2 healthy 'Bonnie Jean' tops +	8 diseased 'Pink Marble' tops	+	+
1 healthy 'Bonnie Jean' top +	9 diseased 'Pink Marble' tops	+	+
	10 diseased 'Pink Marble' tops	+	+

Tabel 1. Het aantonen van chrysantedwergziekteviroïde in mengmonsters van tien toppen van chrysanteplanten, onderzocht met de korte en de volledige PAGE-methode. Elke top woog 1 g. – = geen band; ± = zwakke band; + = duidelijk zichtbare band.

considerable background this would be impossible. In the combinations used, three diseased tops in a total of ten could readily be detected.

Detection of PSTV. In a preliminary experiment using the severe Howell isolate, we tested healthy and diseased potato plants 'Arka' and diseased tomato plants 'Rutgers' and 'Sheyenne' which were grown in a growth chamber (see under Materials and methods). The potato plants had 21 days-old shoots. The tomato plants were harvested 32 days after inoculation. The results of the tests, given in Fig. 4, clearly demonstrate that the diseased potato and tomato plants produced an extra band in the gel as compared to the healthy controls. When these bands were extracted and inoculated on tomato 'Sheyenne' and 'Rutgers', these plants produced the typical PSTV symptoms. Corresponding zones from gels loaded with healthy samples did not produce symptoms.

In another experiment we used healthy and infected potato plants 'Kennebec', grown from the healthy and the diseased tubers supplied by Dr Morris. Healthy 'Sheyenne' plants and those infected by the severe Howell isolate were also used. The infected potato and tomato plants did not show symptoms when they were harvested, four weeks after inoculation of the tomato plants. The infected material produced a typical viroid band upon PAGE. Plants grown in the glasshouse and plants grown in the growth chamber reacted similarly in PAGE. With 'Sheyenne' plants, inoculated with the weak and the severe Howell, the Peters, and the Saco isolates, respectively,

Fig. 4. Electropherograms of healthy and PSTV-infected potato and tomato plants. Healthy (A) and infected (B) potato 'Arka', healthy (C) and infected (D) tomato 'Rutgers' and healthy (E) and infected (F) tomato 'Sheyenne'. V = viroid band.

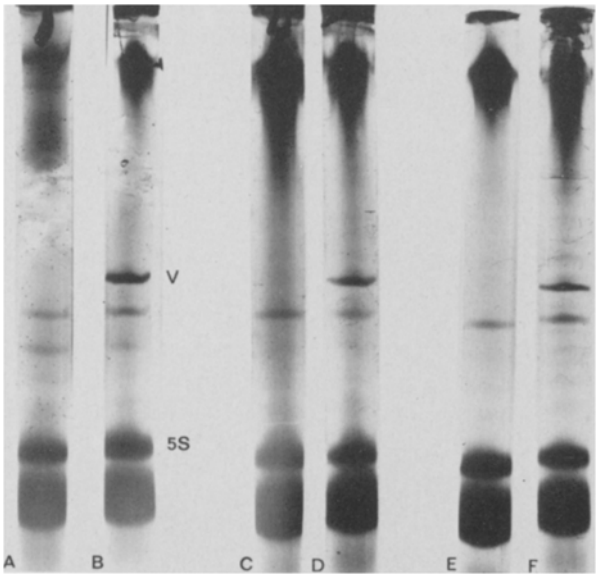

Fig. 4. Elektroferogrammen van gezonde en met PSTV geïnfecteerde aardappel- en tomateplanten. Gezonde (A) en geïnfecteerde (B) aardappel 'Arka', gezonde (C) en geïnfecteerde (D) tomaat 'Rutgers' en gezonde (E) en geïnfecteerde (D) tomaat 'Sheyenne'. V = viroïdeband.

five weeks after inoculation all infections could easily be detected by PAGE. Then, the plants infected with the severe isolate showed symptoms. Symptoms were hardly visible in those with the weak isolates both in glasshouse and growth chamber.

In an effort to detect viroids by PAGE before symptoms appear, tomato plants 'Sheyenne' were inoculated with the four isolates mentioned above, and material was harvested at weekly intervals. All isolates could be detected two weeks after inoculation, even though at that time no symptoms had developed. These appeared first with the severe isolate 18 days after inoculation. In a similar experiment the first detection of PSTV with PAGE was seven days after inoculation, eight days before symptom appearance.

Using sprouts from tubers of 'Kennebec' potato the viroid could be detected in 1.4 g of diseased material. The typical viroid band was not found using healthy tubers.

In one experiment we successfully applied the short PAGE method for detection of the severe Howell isolate of PSTV in tomato plants 'Sheyenne'.

In all combinations of hosts and PSTV isolates tested it was possible to localize the viroid band in the gels by using the $R_m = 0.61$ as described for CSV.

Discussion and conclusion

The nomogram in Fig. 2 could be used for all isolates of the viroids and for all hosts used. In all cases the R_m of the viroid band as compared to the 5S RNA band was 0.61. However, it differed from the R_m that can be calculated from data of Morris and Smith (1977), and which is also independent of viroid isolate and of host. This can be explained by the different temperatures used for electrophoresis which can influence the R_m considerably due to changes in the secondary structure of the RNA.

Our results show that weak and severe PSTV isolates can easily be detected in tomato plants even before symptoms develop. The same holds for shoots of potato plants and for sprouts of potato tubers. Thus, the PAGE method and the biological test on tomato plants 'Rutgers' and 'Sheyenne' are sufficiently reliable under our conditions to detect PSTV in potato.

The screening of chrysanthemums for CSV was as reliable with PAGE as by grafting onto 'Mistletoe' chrysanthemum. Test capacity could be increased in two ways. Sample preparation was shortened to such extent that several samples can be handled per day. Although this gives more background in the gels, it does not reduce test reliability provided tops are tested separately. It was also found that at least ten samples of 1 g each can safely be combined. The method to be selected for routine screening depends largely on the type of material to be tested. In the procedure described dialysis is laborious. The extent of possible mechanization determines applicability for large-scale screening. Moreover, use of slab electrophoresis instead of tube electrophoresis, would further greatly increase the number of samples to be handled per day.

Samenvatting

Het aantonen van het chrysantedwergziekteviroïde en het aardappelspindelknolviroïde met behulp van polyacrylamide-gelelektroforese

Het dwergziekteviroïde (CSV) kon in chrysanten worden aangetoond met een door Morris en Smith (1977) voor het aardappelspindelknolviroïde (PSTV) ontwikkelde polyacrylamide-gelelektroforesemethode (PAGE). Het bereiden van de monsters voor elektroforese kon evenwel aanzienlijk worden vereenvoudigd. De volledige, evenals de korte PAGE-methode bleek even betrouwbaar als de biologische 'Mistletoe'-toets. De PAGE-methode was zo gevoelig dat toepassing ervan op mengmonsters verantwoord is: één besmette top van een chrysantheplant in een totaal van tien kon nog betrouwbaar worden aangetoond.

Hoewel het PSTV niet in Nederland voorkomt, werden de mogelijkheden onderzocht om infecties met dit viroïde te kunnen vaststellen. Kunstmatige infecties met het viroïde in tomate- en aardappelplanten en in aardappelspruiten konden met de door Morris en Smith beschreven PAGE-methode worden aangetoond. Dit gold zowel voor sterke als voor zwakke isolaten, ook als ze geen symptomen veroorzaken. In tomaat kon met de PAGE-methode het PSTV al vier tot acht dagen vóór de symptomen verschenen worden aangetoond.

Acknowledgments

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