Neth. J. Pl. Path. 88 (1982) 113-122

Development of a standard method for detection of potato spindle tuber viroid in potato plants

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Accepted 11 March 1982

Abstract

A standard test method for detecting viroids was designed, to be applied on imported plant material, for which a zero-tolerance exists in the Netherlands towards potato spindle tuber viroid (PSTV).

Partial purification of nucleic acids after homogenizing leaf material with a Polytron homogenizer, followed by increasing the viroid concentration by inoculation of an intermediate tomato host, and complete purification of the small nucleic acids from the tops of these plants, followed by polyacrylamide gelectrophoretic analysis, proved successful. With this procedure, now used as a standard method, more samples could be handled than with other methods tested.

Desalting by Sephadex filtration proved to be superior to dialysis. An attempt to develop a serological test for PSTV failed. Albinism, induced in PSTV-infected tomato plants by certain environmental conditions, was not of diagnostic value.

Additional keywords: potato spindle tuber viroid, chrysanthemum stunt viroid, Sephadex-desalting, serology, viroid-induced albinism.

Introduction

In a previous paper Mosch et al. (1978) reported that the method of Morris and Smith (1977) to detect potato spindle tuber viroid (PSTV) could successfully be used to demonstrate the presence of several isolates of chrysanthemum stunt viroid (CSV) and PSTV. They described how the method could be simplified by grinding the material with an Ultraturrax homogenizer, by omitting the precipitation of large nucleic acid molecules by LiCl, and by omitting the concentration of the viroid RNA by precipitation with ethanol. This method was not sensitive enough to detect the low concentrations of PSTV in imported potato material. The need to test large numbers of samples of imported potato material made it necessary to further simplify the method of Morris and Smith. Moreover, it needed further improvement because in the Netherlands a zero-tolerance exists with respect to PSTV in imported material. Furthermore, it is desirable to detect early infections of PSTV.

Quick methods to test for the presence of PSTV in potato have recently been developed by Pfannenstiel et al. (1980) and L.F. Salazar, Lima, Peru (personal communication). We compared these methods with our method. For the detection of early infections the method of Morris and Smith (1977), using tomato seedlings as an intermediate propagating host, seemed very promising.

Because in the Netherlands testing potatoes for viruses is largely by serology, a serological test for PSTV would fit very well in the routine procedure of the Dutch General Inspection Service. We therefore considered the production of an antiserum to PSTV.

Materials and methods

Preparation of leaf homogenates

Pestle and mortar. Two g of leaf material was homogenized in a mortar in 1 ml of GPS buffer (0.2 M glycine, 0.1 M Na_2HPO_4, 0.6 M NaCl, adjusted to pH 9.6 with 5 M NaOH). Then 4 ml of a mixture of chloroform and 1-pentanol (25:1) were added and the grinding was continued for 5 min.

Pollähne press. Two g of leaf material was crushed between the rollers of the press. The debris was washed into a Corex centrifuge tube containing 4 ml of a mixture of chloroform and 1-pentanol (25:1) with a mixture of 1 ml of GPS buffer and 4 ml of water-saturated phenol containing 7 mM 8-hydroxyquinoline. The mixture was vigorously stirred for 30 sec using a Cenco Whirlmix.

Polytron homogenizer. Two g of leaf material was homogenized in a Corex centrifuge tube in a mixture of 1 ml of GPS buffer, 4 ml of water-saturated phenol containing 7 mM 8-hydroxyquinoline, and 4 ml of a mixture of chloroform and 1-pentanol (25:1) using a Polytron PTA 10-35 homogenizer equipped with a PT 10 generator. It was run at medium speed (PCU control unit at 5) for 30-60 sec.

Test plants

Tomato cv. Rutgers seedlings were grown in a glasshouse at 28-30 °C with continuous additional light of Lux SONT S 400W lamps (producing 240 000 mw/m²) and a relative humidity of 65%. Twelve days after sowing the seedlings were transplanted to 12 l pots filled with sterilized Trio 17 potting soil, each pot containing 4 seedlings. Two days after transplanting, when the seedlings just showed the first true leaf, they were infected by rubbing the inoculum on the carborundum-dusted cotyledons with a cotton swab.

The viroid isolates used in this study were as described earlier (Mosch et al., 1978).

Purification of viroid

Two types of purification were used.

Partial purification. Two g of leaf material was homogenized by one of the methods described above and the homogenate centrifuged for 10 min at 12 000 g. The aqueous supernatant was withdrawn carefully, contamination with material from the lower phase being avoided, and the nucleic acids were precipitated by the addition of two volumes of cold (−20 °C) 96% ethanol and one drop of 4 M sodium acetate. The mixture was stored for 1 h at −20 °C. The sediment formed was precipitated by centri-

fuging for 10 min at 12 000 g. The pellet was resuspended in 0.5 ml of 0.05 M potassium phosphate buffer (pH 7.2).

These preparations were used to inoculate young tomato seedlings, serving as an intermediate propagating host.

'Complete' purification. Leaf material (1g of tomato, 2 g of potato or chrysanthemum) was homogenized as described above. The homogenate was centrifuged for 10 min at 12 000 g. To the supernatant 1/4 volume of 10 M LiCl was added to obtain a final concentration of 2 M. The preparation was stirred thoroughly to dissolve most of the initially formed precipitate, left for 2 h at 4 °C and centrifuged for 10 min at 12 000 g. The supernatant was dialysed against distilled water overnight at 4 °C. Twelve samples were handled simultaneously in a 2 l container. Water was refreshed at least three times during the first 6 h. In some experiments dialysis was replaced by desalting, using Sephadex filtration. After desalting the nucleic acids were precipitated by adding two volumes of cold (−20 °C) 96% ethanol and a drop of 4 M sodium acetate, and storing the mixture for 1 h at −20 °C. the precipitate was collected by centrifuging for 10 min at 12 000 g. The pellet was resuspended in 0.1 ml of autoclaved distilled water.

These preparations were made for analysis by polyacrylamide gelelectrophoresis (PAGE).

Desalting by Sephadex filtration

Pharmacia PD-10 columns, prepacked with 1 g Sephadex G-25M (bed volume 9 ml) were equilibrated with water. The columns were loaded with 2 ml of viroid preparation still containing 2 M LiCl, and the material was eluted with distilled water. The void volume (2 ml) was discarded and the next 3.5 ml, containing most of the viroid, were used for further purification. After washing with 10 ml of 0.1 M NaOH and 50 ml of distilled water the columns could be used again. When, after 30-40 desaltings the columns showed symptoms of blocking, the Sephadex was removed, washed in 0.1% HCl once and in distilled water several times and used to repack the columns.

Polyacrylamide gelelectrophoresis

The nucleic acid preparations were analysed on 5% polyacrylamide gels according to Morris and Smith (1977). Highly purified acrylamide and bisacrylamide were obtained from Bio-Rad Laboratories. Electrophoresis was done on slab gels (7.5 × 7.5 × 0.3 cm). On each slab six samples of 0.04 ml could be analysed and four slabs could be run at the same time in a Pharmacia GE-2/4 electrophoresis apparatus using a Pharmacia EPS 500/400 power supply. The buffer in the electrode vessels was circulated and the apparatus was cooled by running tap water. The gels were prerun for 30 min at a constant voltage of 100 V, which resulted in an amperage of about 50 mA per slab. The samples were electrophoresed under the same conditions until the bromophenol blue tracer dye had almost reached the bottom of the gel.

The nucleic acid patterns were visualized by staining overnight with 0.01% toluidine blue in water followed by destaining in distilled water. In some experiments gels were stained for 15 min in ethidium bromide (20 μg/ml in 1 mM EDTA), destained for 15 min in 1 mM EDTA and examined under UV-light (366 nm).

Serology

Preparations obtained after 'complete' purification of tomato leaf material infected with the severe Howell strain of PSTV, were electrophoresed on slab gels with one big slot. After electrophoresis the viroid band was cut out. The viroid was extracted with distilled water, reconcentrated, and subjected to another electrophoretic purification step. From 500 g of infected plant material 2 ml of viroid preparation was obtained with an $E_{260\,nm}^{1\,cm}$ of c. 2. Each preparation was mixed with 0.5 g of methylated bovine serum albumin before injection into a rabbit. The first preparation was injected intramuscularly. At intervals of 1 week two additional preparations were injected subcutaneously. After two more weeks a fourth preparation, mixed with an equal volume of Freund's incomplete adjuvant, was injected subcutaneously. One month thereafter a blood sample was taken and the titer of the antiserum determined in a micro precipitationtest. In the course of the following 3 months four additional injections of preparations mixed with Freund's incomplete adjuvant were given subcutaneously or intramuscularly, at varying intervals. Altogether the viroid yield of 4 kg of PSTV-infected material was injected.

Results

Development of our standard method. Soon after we started testing imported potato material we found PSTV, if present at all, only in a low concentration. To be sure of the reliability of our tests we then adopted as our standard method a procedure in which the inoculum obtained by partial purification from 2-5 g of leaf tissue was rubbed on four tomato seedlings. If at least one of these plants developed symptoms within 4 weeks, the original sample was considered to be PSTV-infected. If none of the four tomato seedlings of a sample showed symptoms 4 weeks after inoculation, they were subjected to a complete purification, starting from 2 g of material from the top of the plants (0.5 g of each plant) and using Sephadex filtration for desalting. The preparation was analysed by PAGE. Before using this method we investigated some details.

Preparation of leaf homogenates. Grinding leaf material with pestle and mortar was very laborious. To grind 2-5 g of material 10-20 min were needed, and with old or frozen material it took even longer. Furthermore the grinding result was poor when compared with that of power-driven homogenizers.

With a Pollähne press many samples could be handled in a short time and the grinding result was good. However, between samples the press had to be cleaned thoroughly, especially when used in the partial purification to obtain inocula for the intermediate tomato plant. Due to the multiplication of PSTV in this host even the smallest carry-over gave a positive test result. Washing the rollers with a soap solution, followed by rinsing with distilled water, could not fully prevent this.

Grinding with a Polytron homogenizer gave the best results and was very efficient. In the 'complete' purification procedure the homogenizer could be cleaned effectively by running it for 30 sec in running tap water. When used in the partial purification, followed by multiplication of the viroid in tomato seedlings, a more thorough treatment was required. Experiments showed that operating the generator in running tap

water for 30 sec between samples, resulted in a carry-over of 100%. We found the following disinfection procedure satisfactory.

First the generators were rinsed for 30 sec in running tap water, followed by soaking in a soap solution for 30 sec. This was followed by another wash for 30 sec in running tap water before a 5 min soaking in a solution of 0.01 mg RNase-A per ml of 0.01 M sodium phosphate buffer (pH 7.0) at 50-55 °C. Final disinfection was by washing with running tap water followed by heat sterilization for at least 30 min at 120 °C. During the last step the generators were placed in a vertical position, to keep the roller bearings from losing their grease. This procedure fully prevented carry-over.

Test plants. Most of our tests with PSTV were done with tomato cv. Rutgers, although tomato cv. Sheyenne gave similar results. A plant, inoculated when the first leaf just showing, took 10-11 days to develop the first symptoms. Since leaf edges bent downwards the leaves became badly malformed, and the plants became distorted. Such heavily affected plants produced only small amounts of infected material. When the plants were inoculated when about 10 cm high, it took 17 days before the symptoms appeared. However, such plants produced large amounts of infected material, as required for large-scale viroid purification.

Desalting by Sephadex filtration. In order to avoid the laborious and time-consuming dialysis procedure we first tried to omit the LiCl-step. However, in experiments in which both the LiCl-step and the dialysis were omitted, electropherograms were not clear, due to overloading effects or low concentrations. Omitting dialysis only resulted in preparations that, after concentration by ethanol, led to varying nucleic acid patterns in PAGE, due to differences in ionic strength.

The desalting method using prepacked Pharmacia PD-10 Sephadex-G 25 M columns proved to be very efficient. Using 12 columns, 12 samples could be desalted in less than half an hour. Fig. 1 shows a recording of the $E_{254\ nm}^{0.3\ cm}$ of the effluent of a column loaded with 1 ml of sample. The first peak contained the viroid. The second fraction consisted of low molecular UV-absorbing material. In experiments with CSV-infected chrysanthemums and PSTV-infected potato plants we compared desalting by dialysis with desalting by Sephadex filtration and found the latter gave clearer results (Fig. 2). Replacing dialysis by Sephadex filtration made it possible to perform nucleic acid purification, and PAGE in one day. Still the method was laborious. Therefore we compared it with more simple methods of which other research groups claimed good results.

Comparison of our standard method with other testing procedures. The first comparison was made with a method used by L.F. Salazar (personal communication). To increase viroid concentration potato plants obtained from imported tubers were grown for 4 weeks at 26 °C, under continuous light of SONT lamps, and at a relative humidity of 70%. Then leaf samples were taken. After homogenizing them and centrifuging the extract, the supernatant was divided into two parts. One part was subjected to our standard procedure and the other directly to complete purification followed by PAGE, as recommended by Salazar. The results (Table 1) show that our standard method using tomato as an intermediate propagating host in most of the experiments detected more infected samples.

A second comparison, repeated several times, was made with a new electrophoretic

Fig. 1 Separation of PSTV (a) from low molecular UV-absorbing material (b) on a prepacked Pharmacia PD-10 Sephadex G-25 M column. One ml of sample in an aqueous 2 M LiCl solution was applied to the column.

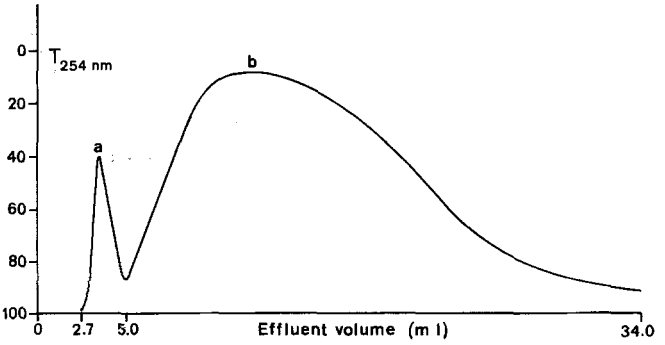

Fig. 1. Scheiding van ASKV (a) van laag moleculair UV-absorberend materiaal (b) via een Pharmacia PD-10 Sephadex G-25 M kolom. Een ml van het monster in een waterige oplossing van 2 M LiCl werd op de kolom aangebracht.

Fig. 2. Electropherograms of tomato extracts, desalted by Sephadex filtration (A, B) or by dialysis (C, D). Extracts were from healthy (A, C) or PSTV-infected plants (B, D).

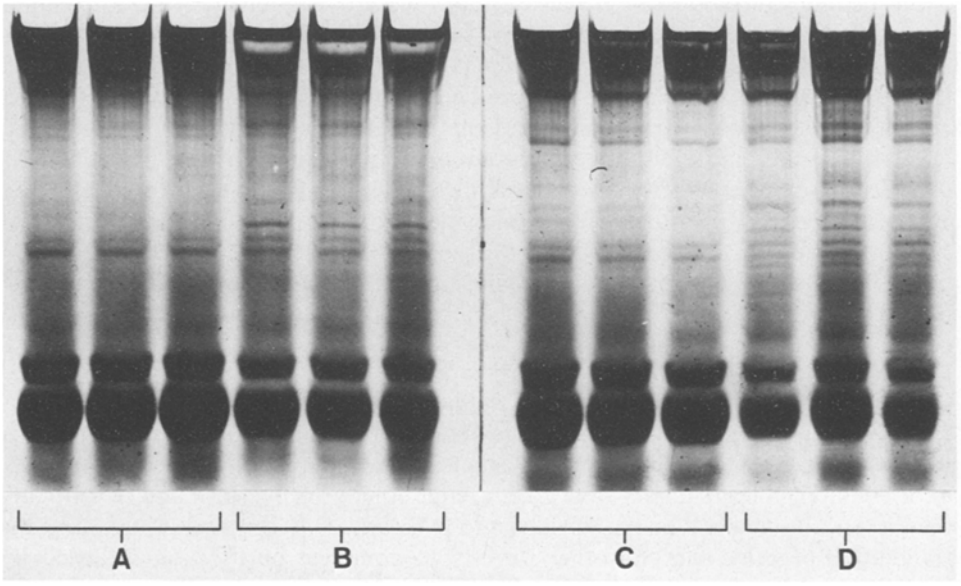

Fig. 2. Elektroferogrammen van tomate-extracten, ontzout via Sephadexfiltratie (A, B) of via dialyse (C, D). De extracten waren bereid van gezonde (A, C) of met ASKV geïnfecteerde (B, D) planten.

Table 1. Detection of PSTV infection, directly from potato plants and via our standard method.

Experiment	Directly from potato[1]	Via standard method[1]
1	0/8	3/8
2	1/18	12/18
3	1/18	7/18
4	1/26	18/26
5	0/14	2/14
6	0/9	0/9
7	1/14	0/14
8	1/9	1/9

[1] Number of plants infected/number of plants tested.

Tabel 1. Het aantonen van ASKV-besmetting direct uit aardappelplanten en via onze standaardmethode.

assay introduced by Pfannenstiel et al. (1980). In this assay LiCl is added directly to the homogenization buffer. After PAGE, ethidium bromide was used to visualize the nucleic acid bands. The test could be completed within one day. L.F. Salazar (personal communication) used this method also with satisfactory results.

Using this method starting from potato leaf material, as well as from tomato plants, PSTV could be detected directly in infected leaves. Often, however, the viroid bands obtained, were less clear than those obtained with our procedure. If leaf homogenates were made with the Pollähne press instead of the Polytron homogenizer, the results were even poorer. Pfannenstiel's method could be improved by desalting by Sephadex filtration. Then the results were similar to those obtained with our method.

In our hands the method of Pfannenstiel et al. (1980) was not succesful for detection of CSV in chrysanthemum.

Visualization of nucleic acid patterns by ethidium bromide or by toluidine blue proved to be equally sensitive in our experiments. Because ethidium bromide is a potentially dangerous chemical, we decided to stain our gels in the standard method with toluidine blue.

Yang and Hooker (1977) observed that tomato cv. Rutgers plants, infected with PSTV showed albinism when grown in soil with a high phosphate level, and kept under continuous light of fluorescent tubes, at a temperature around 29 °C. The diagnostic value of this phenomenon was checked.

Therefore healthy plants and plants infected with the severe and mild Howell strains were grown in growth chambers at 29 °C under continuous cool white light of fluorescent tubes (light intensity at plant level 60 000 MW/m^2). A relative humidity of about 70% was maintained. Of each set of plants half the number were watered with 50 ml of water in the morning and in the evening of every second day. The other plants were given the same amount of water containing 5 g of superphosphate (33% a.i.) per liter. After two weeks the first signs of albinism were seen. However, at the final reading, 3 weeks after infection, albinism was found in some PSTV-infected as well as in some healthy tomato plants. Healthy plants showed it in the older leaves

only. PSTV-infected plants showed albinism in both older and younger leaves. Applying PAGE on inoculated plants free from albinism, all of them were found to be infected with PSTV. The extra supply of phosphate had no significant effect on the detectability of PSTV. Growing plants under the conditions recommended by Yang and Hooker (1977) led to more misreadings of symptoms as compared to growing plants in a glasshouse at 28-30 °C, under continuous light of SONT lamps and at a relative humidity of 70%.

Serology. After four injections the titer of the antiserum was 16, as determined in the micro precipitationtest against a purified viroid preparation. After eight injections the titer was still 16, whereas the titer against purified nucleic acid from healthy material was 4. Using this antiserum in an ELISA test according to Clark and Adams (1977), no difference could be demonstrated between sap from healthy and PSTV-infected tomato leaves.

Discussion and conclusion

Leaf homogenization for viroid extraction was best using a Polytron homogenizer. Using pestle and mortar was more laborious and time-consuming, and the results were inferior. The Pollähne press can handle large numbers of samples and grinding results were good. However, the cleaning of the rollers between samples was difficult and this may form a serious hazard if the press is used in partial purifications for inoculation of tomato seedlings. The Polytron homogenizer worked very conveniently and gave a very good grinding result, which was crucial if the material tested contained a low viroid concentration, as in early infections. If sufficient generators are available one can change generators between samples, which totally prevents carry-over of viroid. Working with 12 generators and cleaning them twice a day, one person can easily handle 24 partial purifications per day.

The conditions in our glasshouse induced good symptoms in tomato and potato. The Yang and Hooker (1977) test did not work in our hands. Albinism appeared in both healthy and PSTV-infected plants, but not in all infected plants. An additional supply of phosphate to the soil did not improve the results, most probably because the Trio potting soil is already rich in nutrients.

We consider desalting of preparations after the LiCl-step in the complete purification a necessary step, because it makes the evaluation of the electrophoretic patterns in the gel easier and more reliable. For small samples this can be done by dialysis or by Sephadex filtration. The latter is now used in our standard method because it was much quicker and gave reliable results. Very few breakdown products were present in the final preparation which resulted in a clear nucleic acid pattern that could be read unequivocally. For large-scale purification, for instance for serology, dialysis was preferred because it is less time-consuming in those cases.

Viroids have a unique structure and form (Sänger et al., 1976). We hoped therefore that this feature would permit the production of a specific antiserum. However, our results indicate that this is not the case. Viroid infections could not be detected serologically in crude sap. Our results are in agreement with those of Stollar and Diener (1971).

Results described in this paper, and the experience of the Dutch Plant Protection

Service using our methods in large-scale testing, indicate that the standard method as described above gives very reliable results. The method requires much time and glasshouse space, but it detects more infected samples than the other methods tested. Samples with low viroid concentration, can also be detected reliably, when an intermediate propagative host is used to enhance the sensitivity of the test. In the Dutch situation, in which a zero-tolerance is used with respect to PSTV in imported material, application of the test described is the best way till now to protect the seed-potato industry against introduction of PSTV.

At present it will be very difficult, if not impossible, to further improve the reliability of the described standard method or to make it less time consuming. New concepts will be needed for more rapid and reliable tests for viroids. The c-DNA hybridization test of Owens and Diener (1981) may well prove to be one of these.

Acknowledgement

We are greatly indebted to Lenie van Erk, Greet de Raay and Mirjam Slaa for technical assistance.

Samenvatting

Ontwikkeling van een standaardmethode voor het aantonen van aardappelspindelknolviroïde in aardappelplanten

Voor het viroïde, dat de aardappelspindelknolziekte veroorzaakt (ASKV) geldt in Nederland een nultolerantie. Al het geïmporteerde aardappelmateriaal wordt daarom getoetst op het voorkomen van het viroïde. Voor dat doel is een betrouwbare en, zo mogelijk, ook snelle toets noodzakelijk, die niet alleen secundaire infecties maar ook jonge, primaire infecties kan aantonen.

Een standaardmethode, die werd ontwikkeld, bleek zeer betrouwbaar, hoewel niet snel. Zij toont meer infecties aan dan snellere methoden die in het buitenland beschreven zijn. De toets omvat de volgende stappen: 2-5 g bladmateriaal wordt vermalen en op het homogenaat wordt een eenvoudige nucleïnezuurextractie en -concentratie toegepast. Dit preparaat wordt gebruikt om vier jonge tomatezaailingen te inoculeren. Door deze zaailingen 4 weken onder optimale omstandigheden te houden wordt het eventueel aanwezige viroïde vermeerderd. Geeft tenminste één van de tomateplanten symptomen, dan wordt het oorspronkelijke monster ziek verklaard. Vertoont geen van de vier tomateplanten symptomen dan wordt een nucleïnezuurextractie uitgevoerd van de topjes van deze planten. Kleine nucleïnezuurmoleculen worden geïsoleerd, geconcentreerd en tenslotte geanaliseerd met behulp van polyacrylamide gelelektroforese.

Om overdracht van het ene naar het andere monster te voorkomen werd voor het vermalen gebruik gemaakt van verwisselbare schachten bij de Polytron homogenisator.

Ontzouten van de nucleïnezuurextracten met Sephadexfiltratie gaf betere resultaten en was sneller uitvoerbaar dan dialyse.

Pogingen om een specifiek antiserum tegen ASKV te maken zijn niet gelukt. Onder onze omstandigheden was het ook niet mogelijk om op een betrouwbare manier albi-

nisme in geïnfecteerde planten te induceren als middel om infecties met ASKV op te sporen.

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