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Detection of Important Plant Viruses in *In vitro* Regenerated Potato Plants by Double Antibody Sandwich Method of ELISA

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Abstract

To develop a reliable and sensitive method for virus identification *in vitro* regenerated plantlets of six potato varieties such as, Cardinal, Diamant, Dhera, Multa, Cilena and Sieglinde were mechanically inoculated with seven potato viruses, namely A, Y, V, M, S, X and PLRV and identified through double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA). *In vitro* regenerated non-inoculated plants were used as negative control. Among the seven viruses used, except PLRV others infected the host tissue mechanically. Although no physical symptoms could be observed in some cases after mechanical infection, the presence of viruses could be detected through DAS-ELISA test. There were no antiserum reactions in the non-infected and *in vitro* regenerated negative control plants.

Introduction

Potato (*Solanum tuberosum* L.) species are host to the largest number of viruses. At least 37 viruses naturally infect cultivated potatoes (Beemster and de Bokx, 1987; Salazar 1996; Jeffries 1998). Some of these viruses, notably potato leaf roll (PLRV), potato virus A (PVA), potato virus Y (PVY), potato virus V (PVV), potato virus M (PVM), potato virus X (PVX), potato virus S (PVS), potato mop top virus (PMTV) and potato aucuba mosaic virus (PAMV) occur worldwide in potato crops; others are important only in some geographical areas (Brunt 2001). It is well-known that it is possible to develop virus free potato plants through *in vitro* meristem culture (Faccioli 2001). It is, however essential to ensure that the supplied *in vitro* regenerated plants or microtubers are virus free for commercial potato cultivation.

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There are a number of systems available to detect viruses (Singh 1999). Many certification authorities prefer visual detection of pathogens on the potato plants or seed tubers. Relying on such visual detection of symptoms is, however not always conclusive. In many cases plants may carry a latent infection which does not produce detectable symptoms, as with certain strains of potato virus X (Banttari et al. 1993). Serological procedures form the most reliable method for identification and quantitative assay of viruses. In this context the direct double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) is used widely for virus detection (Clark and Adams 1977). The advantage of this assay is that the virus particles are concentrated from extracts by the coating antibody, and inhibitory components of extracts are removed by rinsing before addition of detecting antibody and enzyme substrate.

In the present investigation we have studied the presence or absence of the virus particles in mechanically infected *in vitro* regenerated plants with seven viruses, namely PLRV, PVA, PVY, PVV, PVM, PVS and PVX using DAS-ELISA.

Materials and Methods

Six cultivated potato (*Solanum tuberosum* L.) varieties of which four, namely Cardinal, Diamant, Dhera and Multa were collected from Bangladesh and two *viz.* Cilena and Sieglinde were from Germany.

Collection of plant samples : Leaf samples were collected from *in vitro* raised plants of all varieties which were grown in the greenhouse, in non-infected (S1) and infected (S2) chambers. Field samples were collected from the plants showing virus-like symptoms as well as from symptomless ones from Saka-Ragis Pflanzenzucht GbR experimental field, Zuchtstation Windeby 24304, Windeby bei Eckernforde, Germany.

Source of ELISA kits : Antiserum, antibody conjugated with alkaline phosphatase (IgG conjugate) and positive control for PLRV, PVA, PVY, PVV, PVM, PVS and PVX were collected through the courtesy of Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), C/o. Biologische Bundesanstatt Institut fur Pflanzenvirologie, Mikrobiologie und Biologische Sicherheit, Messeweg 11/12, D-38104 Braunschweig, Germany.

Inoculation of potato plant with viruses : The *in vitro* raised plantlets and microtubers were sown in pots and as well as grown in the greenhouse. Two weeks after sowing, the plantlets were mechanically inoculated at their primary leaves with a crude leaf homogenate of positive control plants infected with PLRV, PVA, PVY, PVV, PVM, PVS and PVX. For mechanically

inoculation known infected leaves were first ground in a buffer (0.01M sodium phosphate containing 0.4% sodium sulphite, pH 7.5) and small amount sea sand to provide a 1 : 5 dilution of sap. The homogenate was manually spread on the dorsal surface of leaf by gloved-fingers. A total of 84 plants two each from six varieties were infected with the afforesaid seven viruses. The plants were grown in greenhouse at a constant temperature of 26_C with additional supply of 4,000 lux for 12 h provided by Na-High-Pressure lamps, type PL780/N400 (Poot Lightenerge, The Netherlands).

DAS-ELISA : It was performed following the protocols described by Clark and Adams (1977). Microtitre plate wells (8 ∇ 12 flat-bottom wells of c. 400 µl/well) were coated with antiserum diluted in carbonate buffer (pH 9.6) according to the supplier's specifications. IgG used in the present experiment was diluted in the carbonate buffer in the proportion of 1 : 1000. Plates were incubated for 3 h at 37_C. Following incubation plant extracts were applied on these wells. The plant extracts were prepared with a hand-held rotary grinder in a maceration buffer containing phosphate buffered saline (PBS) with 0.5 ml/l Tween 20 and 2% polyvinyl pyrrolidone. After adding the crude plant extract, the virus was detected by the corresponding antibody conjugated with alkaline phosphate diluted in conjugate buffer (PBS-TPO, pH 7.4) according to the suppliers specifications.

Plates were washed with phosphate buffered saline (PBS, pH 7.4) at each stage. Absorbance at 405 nm (measured with a ELISA Reader; DYNARECH MR 50000, U.S.A.) was read 30 min to 3 h after incubation with the substrate p-nitrophenyl phosphate (1 mg/ml, pH 9.8). A sample was considered positive if the absorbance was at least three times greater than that of the healthy control plant (negative control).

Results and Discussion

The main objective of the present investigation was to develop a reliable and sensitive protocol to detect the potato viruses in *in vitro* regenerated plants. For this purpose all the six varieties used in the present investigation were mechanically inoculated with the seven potato viruses, namely PLRV, PVA, PVY, PVV, PVM, PVS and PVX. A total of 84 plants (12 for each virus) were used for infection of all the six varieties and monitored over three, four, five and six weeks after inoculation. Non-inoculated *in vitro* regenerated plants were used as negative control whereas the plant parts infected with known viruses were used as positive control. The host-pathogen interaction for all the viruses and their detection on the host plants have been separately presented Table 1.

Table 1. The host-pathogen interaction for seven potato viruses following mechani-cal inoculation of *in vitro* grown plants.

Name of the viruses	No. of mechanically inoculated plants	No. of infected plants	Days of first virus infection	Symptoms developed following infection	DAS-ELISA reaction
PLRV	12	0	-	Symptomless	No antiserum reaction
PVA	12	12	21	Mild mosaic, thick and wrinkled leaves, marginal distortion	Strong antiserum reaction
PVY	12	8	28	Leaf mottling mosaic, necrosis	Antiserum reacted with symptomized plants
PVV	12	8	21	Slightly whitish, smaller leaves, necrosis	Antiserum reacted with infected plants
PVM	12	12	28	Mildly mosaic pattern, crinkling, serious necrosis	Antiserum reacted strongly
PVX	12	12	21	Mild leaf mosaic pattern, crinkling, thick and wrinkled leaves	Antiserum reacted
PVS	12	12	28	Symptomless	Strong antiserum reaction

PLRV: This virus did not produce any symptoms on the *in vitro* regenerated plantlets even six weeks after inoculation. However, viral symptoms like pallor and upward rolling of young leaves, specially at the base, stunted plant growth habit and yellowing of leaf margins were recorded from the field grown plants collected from Saka-Ragis Pflanzenzucht GbR, Windeby, Germany (Fig. 1A). Rodriguez and Jones (1978) also observed similar responses from field grown potato plants. The presence of PLRV was confirmed by DAS-ELISA test where antiserum reacted strongly in the plants collected from the field but not in *in vitro* grown plants (Table 1). The positive control plants (obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Braunschweig, Germany) also produced similar results. The result indicated that PLRV is not transmitted by mechanical inoculation and *in vitro* plants were PLRV free. Liza'rraga et al. (1991) produced PLRV free plants through tissue culture.

PVA : Among the six potato varieties infected, a mild mosaic, thick, wrinkled and marginal distorted leaves were observed in the variety Sieglinde three weeks after inoculation (Fig. 1B). The other five varieties developed PVA symptoms after four weeks of inoculation. In the DAS-ELISA test

antiserum reacted strongly with the PVA infected plants whereas nontreated plants did not show any reaction (Table 1). The above result indicated that PVA can be transmitted by mechanical inoculation and variety Sieglinde is more sensitive to PVA than other varieties.

Fig. 1 A-F: Symptoms developed by different viruses. A. Potato leaf roll symptoms by PLRV. B. Mild mosaic and rugosity symptoms by PVA. C. Leaf mottling and mosaic symptoms by PVY. D. Pallid and small leaves symptoms by PVV. E. Photograph showing crinkling and necrosis symptoms by PVM. F. Mild mosaic, crinkling and rugosity symptoms by PVX.

PVY : A total of 12 plants from six varieties were mechanically infected with PVY. Symptoms like leaf mottling mosaic and necrosis were observed in eight plants four weeks after inoculation (Fig. 1C). This result indicated that PVY cannot always produce physical symptoms. It has been shown through the DAS-ELISA test that antiserum reacted strongly with infected plants (Table 1). Morel et al. (1968) obtained PVA and PVY free potato plants through *in vitro* culture using less than 0.3 mm meristem in variety Kelopetra. There are some reports that PVY can be occasionally transmitted mechanically by inoculation (Barker et al. 1993; Jeffries 1998).

PVV : Among the six varieties used for mechanical infection with PVV, symptoms like slightly whitish, production of smaller leaves and necrosis were observed only in var. Diamant three weeks after inoculation (Fig. 1D). However, the other five varieties did not show any symptom even after a long period of time. Although these varieties (Cardinal, Dhera and Multa) did not show any symptoms under visual observation, they showed positive reaction with antiserum during the DAS-ELISA test (Table 1). However, PVV could not infect the German varieties, namely Cilena and Sieglinde. The above findings indicated that PVV can also be transmitted mechanically and can produce symptoms in some members restricted within the family Solanaceae. Previous workers also observed similar results in case of potato variety Adretta (Fribourg and Nakashima 1984; Jones and Fuller 1984).

PVM : Mildly mosaic patterns, crinkling and serious necrosis of leaves were observed in all the infected varieties four weeks after inoculation with PVM (Fig. 1E). In the DAS-ELISA test antiserum reacted only with the infected plants and as expected did not show any reaction with non-infected and *in vitro* regenerated control plants (Fig. 2; lanes A, B, C). This result proved that PVM is mechanically transmissible and was highly infectious to all the varieties used in the present investigation (Table 1). Faccioli and Colombarini (1996) observed similar results and detected the presence of PVS and PVM contents in potato meristem tips.

PVX : The symptoms of plants infected with PVX were the presence of mild leaf mosaic pattern, crinkling, thick and wrinkled of leaves. These symptoms appeared three weeks after inoculation in all the six varieties (Fig. 1F). The antiserum reacted strongly with all infected plants by the DAS-ELISA test (Fig. 2, lanes E, F, G). It may be mentioned that all the six varieties were most sensitive to PVX compared to other viruses tested in this investigation (Table 1). Koenig (1988) also observed similar results for the detection of potato viruses through serological tests.

PVS: All the six varieties used in this experiment did not show any symptom after mechanical infection with PVS. However, in the DAS-ELISA test all the infected plants showed strong positive reaction with antiserum after four weeks of inoculation (Table 1). Similar findings were reported earlier by Schiessendoppler and Forchum (1990) using dot ELISA. These results indicated that PVS can be transmitted through mechanical inoculation and all the six varieties are susceptible to PVS (Salazar 1996).

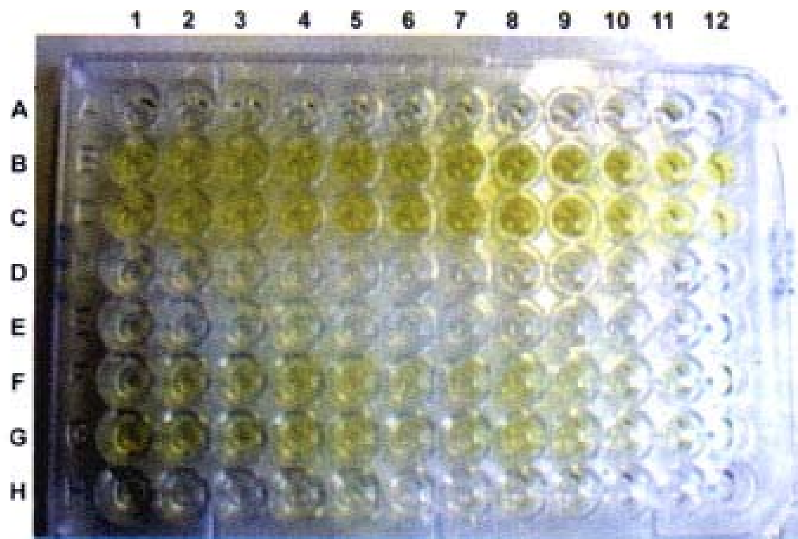

Fig. 2: DAS-ELISA test for PVM and PVX (Yellow cavities indicate the presence of virus and clear cavities indicate the absence of virus). Lane A: non-treated plants for PVM test, lanes B & C: leaf extracts from mechanically infected plants of PVM except cavities 11 and 12 of lane C which is used as positive control. Lane E: non-treated plants for PVX test, lanes F & G: leaf extracts from mechanically infected plants with PVX except cavities 11 and 12 of lane G which is used as positive control. Lanes D & H: blank.

In developing countries, virus diseases are of major concern which lowers the yields of potato and requires the development of appropriate, sensitive and reliable detection methods (Salazar 1994). Control of potato viruses is difficult because, unlike pathogenic microorganisms, viruses are unaffected by therapeutic treatments; their spread is thus best minimized by preventive measures. As potato is a vegetatively propagated crop, infection increases through subsequent generation. The most effective approaches to control viruses in potato include the development of sensitive, simple and economical detection methods in any particular region.

The results of the present study indicate that it is possible to detect PLRV, PVA, PVY, PVV, PVM, PVS and PVX by the DAS-ELISA test from *in vitro* raised infected and non-infected plants and it may be recommended that the above DAS-ELISA method can be widely used for virus detection in potato and other related plants. As already mentioned the advantage of this assay is that the virus particles are concentrated from extracts by the coating antibody, and the inhibitory components of extracts are removed by rinsing before addition of detecting antibody and enzyme substrate. The limit of detection of most plant viruses in tests on host tissue extracts between 1 and 10 ng/ml when using the chromogenic substrate p-nitrophenyl phosphate (NPP) (Regenmortel 1982). The product of hydrolysis of NPP by alkaline phosphate (AP), turns yellow in alkaline solution and strongly absorbs light at 405 nm. Substantially lower limits of detection (>100 ∇) can be achieved by using fluorogenic or chemiluminescent substrates.

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