

## PRODUCTION OF DIAGNOSTIC ANTIBODIES TO PLANT VIRUSES, UTILIZING DENATURED COAT PROTEINS AND BOOSTER IMMUNIZATION WITH PARTIALLY PURIFIED VIRUS

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### SUMMARY

Groups of rabbits were immunized intramuscularly with either partially purified or denatured Iris Yellow Spot Virus (IYSV) nucleocapsid protein (NC) excised from SDS-polyacrylamide gels. Four injections were given at weekly intervals. Two weeks later, booster injections were given either with partially purified preparations of native virions or with denatured NC. Twelve days after the booster, rabbits were bled and the binding capacity of antisera was examined by decoration tests, Western blotting and ELISA. Diagnostic antibodies which could specifically bind to virus particles were obtained. Higher ELISA values were obtained when the booster injection contained partially purified IYSV, rather than denatured NC. Similar results were obtained with Potato Virus Y.

### RIASSUNTO

**ANTICORPI DIAGNOSTICI PER FITOVIRUS OTTENUTI CON L'USO DI PROTEINA CAPSIDICA DENATURATA E IMMUNIZZAZIONE DI RICHIAMO CON VIRUS PARZIALMENTE PURIFICATO.** Sono stati immunizzati conigli con quattro iniezioni intramuscolari settimanali della proteina nucleocapsidica del tospovirus della maculatura gialla dell'iris (IYSV) parzialmente purificata o denaturata, seguite a due settimane da un'iniezione di richiamo con preparati parzialmente purificati di virus o con proteina nucleocapsidica denaturata. A dodici giorni di distanza i conigli sono stati salassati e l'efficacia degli antisieri è stata verificata con saggi di immunomicroscopia elettronica (decorazione), Western blot ed ELISA, dimostrando che gli anticorpi prodotti riconoscono specificamente le particelle virali. I più elevati valori ELISA sono stati rilevati con antisieri in cui l'immunizzazione di richiamo era stata effettuata con virus purificato più che con proteina nucleocapsidica denaturata. Risultati analoghi sono stati ottenuti col virus Y della patata (PVY).

*Key words:* Iris Yellow Spot, Potato Virus Y, diagnostic antibodies, coat protein, decoration, ELISA, immunoblot.

### INTRODUCTION

Accurate detection of plant viruses is essential for preventing their spread to new areas. Although enzyme-linked immunosorbent assay (ELISA) is used routinely to detect viruses and in resistance breeding programs, a major limitation appears to be the availability of high quality antisera. In order to produce antibodies to viral proteins by conventional methods, purification of the virus is required. It is often difficult to obtain purified preparations not contaminated with healthy plant proteins (Brunt and Lawson, 1995), so, polyclonal antisera often contain nonspecific antibodies which react with healthy plant extracts (Van Regenmortel, 1982). Additionally, elaborate facilities are required for virus purification (Brunt and Lawson, 1995).

Antisera to viral coat proteins have been obtained by immunization with viruses degraded by sodium dodecyl sulfate (SDS). Small amounts of protein obtained from bands cut from gels after electrophoresis have also been found to yield antisera of adequate titre (Caroll *et al.*, 1978).

Recently we have shown that booster immunization with a partially purified Citrus Tristeza Virus (CTV) preparation after priming with recombinant CTV coat protein enhances the binding capacity of capture antibodies as measured by ELISA (Bar-Joseph *et al.*, 1997). Here we report the application of this method for the production of diagnostic antibodies to SDS-polyacrylamide gel electrophoresed Iris Yellow Spot Virus (IYSV) and Potato Virus Y (PVY).

### MATERIALS AND METHODS

**Virus purification.** IYSV originally isolated from field-grown onion (*Allium cepa*) (Gera *et al.*, 1998), was propagated in *Nicotiana benthamiana* and partially pu-

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rified by the method described by Avila *et al.* (1990).

PVY was partially purified from *N. glutinosa* according to Moghal and Francki (1976), and further purified by layering the preparation on a linear sucrose gradient (10-40% in 0.05 M sodium borate buffer, pH 8.8, containing 0.01 M EDTA) (Zeidan *et al.*, 1998). Fractions containing the highest concentration of virus were pooled and centrifuged.

**SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting.** Samples from PVY and IYSV-enriched preparations were electrophoresed in SDS-PAGE according to Laemmli (1970). Polyacrylamide gel (12%, 75 x 5 mm) were prepared. Proteins used as size markers were: phosphorylase b (97.4 kDa), bovine serum albumin (66.3 kDa), aldolase (42.4 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and lysozyme (14.4 kDa). The protein bands were stained with either 0.1% Coomassie Brilliant Blue or electroblotted to nitrocellulose (Towbin *et al.*, 1979) and probed with homologous antiserum. Antibody binding was detected using anti-rabbit IgG conjugated to alkaline phosphatase (BioMakor, Rehovot, Israel).

**Production of antibodies.** Partially purified virus (ppV) suspensions of IYSV and PVY (about 0.1 mg ml<sup>-1</sup>) were emulsified with an equal volume of Freund's incomplete adjuvant and injected intramuscularly (IM) into both hip muscles of 6 week-old female New Zealand white rabbits. Four IM injections were given at weekly intervals. Two weeks after the last injection a booster intravenous (IV) injection with ppV was given. Such antisera (designated ppV/ppV) were collected 12 days after boosting. Alternatively, the proteins from purified virus preparations were denatured with SDS and separated by SDS-PAGE as described previously (Zeidan *et al.*, 1998). Stained protein bands (SDS-PB) were excised from the gel, washed, crushed in 1 ml of 0.01 M phosphate buffer, pH 7, and emulsified with Freund's complete or incomplete adjuvant for the first and subsequent injections, respectively. Rabbits were given four IM injections at weekly intervals. Two weeks after the last injection, a booster injection with ppV or SDS-PB was given. Antisera were again collected 12 days after boosting. These antisera are designated SDS-PB/ppV and SDS-PB/SDS-PB, respectively. All antisera were tested using Western blotting (Towbin *et al.*, 1979), ELISA (Clark and Adams, 1977) and by decoration tests as described by (Milne and Luisoni, 1975).

## RESULTS AND DISCUSSION

Purified IYSV proteins, when denatured with SDS and analyzed by PAGE, gave three sharp bands, which by analogy with TSWV, were G1 (78 kDa), G2 (58 kDa) and the nucleocapsid protein (NC) (31 kDa), respectively (Fig. 1A). Immunoblots with SDS-PB/ppV and ppV/ppV of IYSV, gave a clear and strong reaction with the polypeptides corresponding to NC protein as identified by their molecular weight (Fig. 1B, 1C). Although both IYSV antisera reacted with the NC protein, the reactivity and specificity of the SDS-PB/ppV antisera were higher. Similar results were obtained with PVY (results not shown).

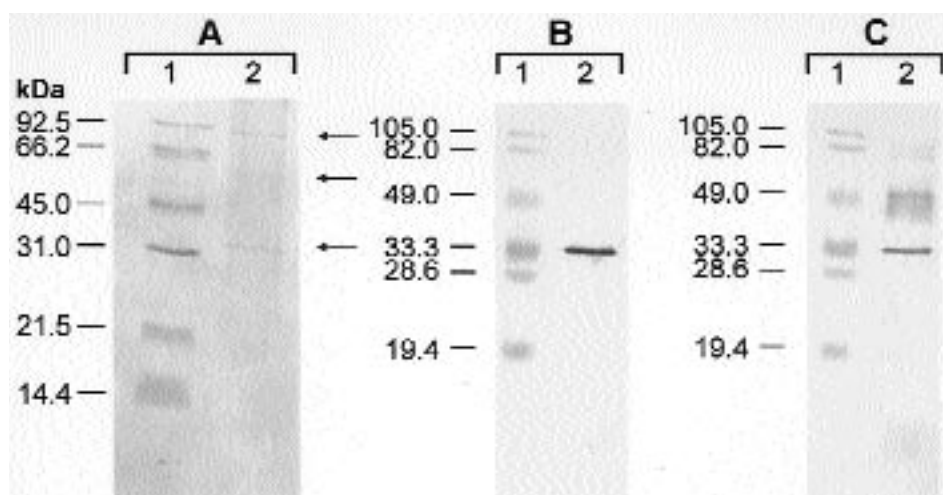
ELISA titres (OD/405 nm) with SDS-PB/ppV antisera against IYSV and PVY are summarized in Table 1. Considerably higher titers were obtained with the SDS-PB/ppV antisera than with ppV/ppV antisera, for both viruses. The reaction of ppV/ppV antisera against IYSV with *N. benthamiana* infected tissue in DAS-ELISA was weak and gave a high unspecific background with healthy plant extracts (Table 1). SDS-PB/SDS-PB antisera against IYSV did not react with IYSV infected tissues in DAS-ELISA (results not shown).

Decoration tests with SDS-PB/ppV antisera against PVY resulted in strong decoration of the particles (Fig. 2).

The polyclonal antibodies produced against the two viruses were compared for their ability to detect the viruses in infected plant material. SDS-PB/ppV antisera against IYSV and PVY were highly specific and more efficient in detecting the virus even when infected plant material was used at a dilution of 1:100,000, whereas the ppV/ppV antisera produced against both viruses failed to detect the virus at dilutions beyond 1:1000. These antibodies evinced a nonspecific reaction with healthy tissue. It is well known that the injection and bleeding schedule as well as the preparation of antigen, can greatly affect serum titer (Van Regenmortel, 1982). Our results indicate the considerable advantage of using primary injection and boosting with gel separated and native antigens, respectively, to obtain higher titer antisera suitable for ELISA.

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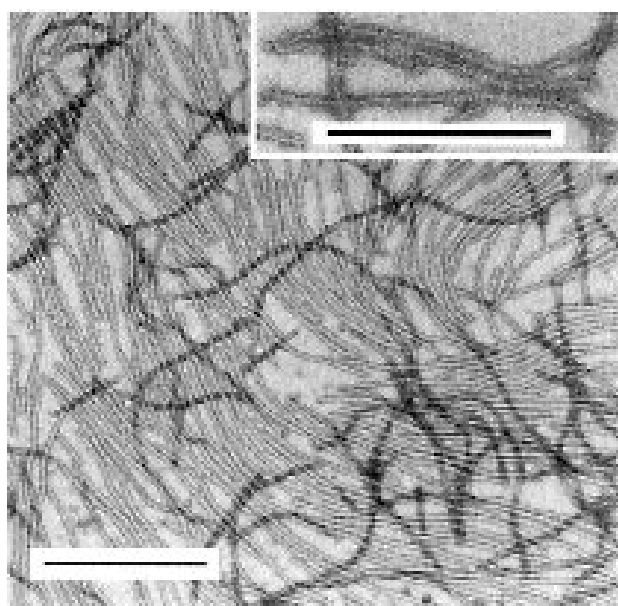


**Fig. 1.** SDS - polyacrylamide gel electrophoresis of Iris Yellow Spot Virus (IYSV). Lane 1: molecular weight markers; Lane 2: partially purified preparation stained with Coomassie Brilliant Blue (A) and Immunoblots probed with polyclonal antibodies (diluted 1:2000) obtained after primary injections with SDS-denatured protein bands (SDS-PB) (B), or partially purified viral (ppV) antigen (C) and boosting with partially purified viral antigen.

**Table 1.** ELISA values (OD/405 nm) of Iris Yellow Spot Virus (IYSV) from infected *Nicotiana benthamiana* and Potato Virus Y (PVY) from *N. glutinosa* with antibodies obtained after primary injections with either partially purified viral antigen (pp) or SDS-denatured protein bands (SDS-PB) and boosting with ppV.

Virus	Injection route	OD of diluted leaf samples			
		1:10		1:100	
		infected	healthy	infected	healthy
IYSV	SDS-PB/ pp	1.85±0.22	0.08±0.04	1.28±0.19	0.06±0.03
	pp/ pp	0.81±0.08	0.28±0.16	0.55±0.06	0.24±0.14
PVY	SDS-PB/ pp	1.68±0.16	0.08±0.05	1.36±0.11	0.05±0.03
	pp/ pp	0.95±0.12	0.30±0.20	0.75±0.05	0.25±0.18

\* Average of 4-6 replicas per sample and standard deviation. Each experiment was repeated three times when IgG and conjugate concentration were 1:4000 and 1:2000 for IYSV and PVY, respectively.



**Fig. 2.** Electron micrograph of purified particles of Potato Virus Y (PVY) stained with uranyl acetate after decoration with polyclonal antibodies (diluted 1:50). The antibodies were obtained after primary injections with SDS-denatured protein bands and boosting with partially purified PVY (insert-right top). Bars = 500 nm.

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