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FACTORS INFLUENCING IMMUNE ELECTRON MICROSCOPY OF FLEXUOUS POTATO VIRUSES

I. D. GARG, S. M. PAUL KHURANA

Central Potato Research Institute, Shimla 171 001, India

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Summary. – Effects of pH of extraction buffers, pH and titer of trapping antisera and their combinations, virus acquisition time and virus host on the trapping efficiency of flexuous potato viruses X, S and Y (PVX, PVS and PVY) in immune electron microscopy were evaluated. Addition of ethylene-diamine-tetraacetic acid to the extraction buffer improved trapping of PVY, adversely affected PVS but not PVX. Combinations of antisera had differential adverse effect on trapping which was maximum with the mixture of three antisera. Mixture of antisera to PVX and PVY had the least adverse effect on trapping of PVX and PVY as compared to the mixture with PVS antiserum. Trapping of PVX and PVY was good and almost at par at all the dilutions of the antisera while that of PVS was good up to 1000-fold only. Prolonged virus acquisition time significantly increased the number of virions trapped. Trapping was affected both by the pH of the antiserum and the extraction buffer, while in the case of PVY it was also affected by the host species.

Key words: PVX; PVS; PVY; buffers; antisera combinations; immune electron microscopy

Introduction

Immune electron microscopy (IEM) is a well documented and a highly sensitive virus detection technique (Milne and Luisoni, 1977; Khurana and Garg, 1989). The technique has also been found to be highly effective for potato viruses (Roberts and Harrison, 1979; Braun and Opgenorth, 1987; Garg and Khurana, 1988; Singh *et al.*, 1990). Factors like type and pH of the extraction buffer, antiserum dilution and its pH, incubation period for virions trapping (virus acquisition time, VAT) and pre-treatment of the grid with protein-A and ethidium bromide (Balen, 1982; Cohen *et al.*, 1982; Milne, 1986; Pares and Whitecross, 1985; Shukla and Gough, 1979) influence the efficiency of IEM. They affect the efficiency of IEM differently for different viruses (Cohen *et al.*,

1982). This paper reports results of the studies on the effect of extraction buffers pH as well as of trapping antisera, VAT, antisera combinations and host species on trapping efficiency of three common flexuous potato viruses, PVX (potexvirus), PVS (carlavirus) and PVY (potyvirus).

Materials and Methods

Virus extracts. Pure cultures of PVX, PVS and PVY° maintained in *Nicotiana glutinosa*, potato cv. Craig's Defiance and *N. tabacum* cv. Havana, respectively at Central Potato Research Institute, Shimla were used. Besides, potato cv. Kufri Chandramukhi and *Datura metel* were also inoculated with PVY°. Crude virus extracts were prepared by homogenizing 0.5 g of infected leaf tissue with 4 ml of the buffer in a mortar with pestle. The slurry thus obtained was diluted with the buffer and then passed through a loose was of cotton wool and the filtrate served as the crude virus extract.

Buffers. Two buffers, namely Sorensen's phosphate buffer (PB) (0.1 mol/l, pH 7.2) alone and the same supplemented with 0.1 mol/l disodium-ethylenediamine-tetracetic acid (PB+EDTA) were used to prepare the virus extracs. Besides these, PB extraction buffers pH 6.0 and 8.0 were also used.

Antisera. Polyclonal antisera to purified PVX, PVS and PVY° (Khurana *et al.*, 1987; 1990), raised in rabbits had initial microprecipitin titers of 1:2000, 1:512 and 1:512, respectively. PVX antiserum was first diluted 1:4 with 0.85 % saline to make it equal to those of PVS and PVY. Then all the antisera were diluted 1:600, 1:1000 and 1:1600 with saline for use.

Coating of grids with antisera and virion trapping. Colodion-copper grids were coated with different dilutions of individual antisera as well as their combination by floating on 20 μl drops for 20 min followed by washing with 30 drops of distilled water and draining briefly by touching the grid edge with filter paper. Trapping of virions was carried out by immediate transfer of antisera coated grids onto 20 μl drops of crude virus extracts. In all cases, unless otherwise stated, the reaction proceeded for 1 hr at 37 °C in humid Petri dishes. Grids were then washed and drained as mentioned above and negatively stained with 2 % aqueous uranylacetate.

Effect of buffers. Virus extracts used were prepared in the two buffers (PB and PB+EDTA) at a final dilution of 1:2000 for PVX, and 1:30 for PVS and PVY. Grids coated with 1:1000 diluted individual antisera were used for trapping as already described.

Effect of pH of antiserum and extraction buffer. PVX and PVS extracted in PB and PVY in PB+EDTA, pH 6.0, 7.2 and 8.0 at final dilution (w/v) of 1:2000 for PVX and 1:30 for PVS and PVY were used. Grids were coated with 1:1000 diluted antisera pH 6.0, 7.0 and 8.0.

Effect of antisera combinations. PVX and PVS extracted in PB pH 7.2 and PVY in PB+EDTA pH 7.2 were used. Combinations of antisera for coating the grids were PVX+PVS, PVX+PVY, PVS+PVY and PVX+PVS+PVY. Each combination had the same three dilutions of 1:600, 1:1000 and 1:1600 (i. e. individual antisera in the mixtures had these dilutions).

Effect of simultaneous trapping for more than one virus. Virus extracts prepared as described in preceding paragraph and their mixtures i. e. PVX+PVS, PVX+PVY, PVS+PVY and PVX+PVS+PVY having the same individual virus concentration (1:2000 for PVX and 1:30 for PVS and PVY) were used. Trapping was done by coating grids with individual antisera as well as their combinations where dilution of each antiserum was maintained at 1:1000.

Effect of VAT. Grids coated with individual antisera diluted 1:600 and 1:1000 were used to trap virions. Virus extracts used had a final dilution of 1:4000 for PVX (with PB), 1:100 for PVS (with PB) and PVY (with PB+EDTA). VAT was 1, 6 and 24 hr. Grids were incubated for 1 hr at 37 °C and then transferred to 4 °C for 6 hr and 24 hr.

Data recording. In each experiment, virus particles were counted at a displayed magnification of 21 000 x on a fluorescent screen (5x4 cm). Ten counts from each grid square were made (Cohen *et al.*, 1982) and their mean calculated. Fifteen such means were determined from 15 squares of three grids per treatment. The grand mean did not differ significantly from individual means.

Results

Effect of buffers

The results (Table 1) revealed that extraction of PVY with PB caused aggregation of virions while PB supplemented with EDTA prevented it and resulted in uniform distribution of virions on the grid. However, addition of EDTA to PB for the extraction of PVX had no effect both on trapping and distribution of virions. On the contrary, trapping and distribution of PVS was better with PB alone and adversely affected when PB had been supplemented with EDTA.

Effect of pH

Results on the effect of pH of the extraction buffer as well as that of the antiserum on trapping of virions are given in Fig. 1. Trapping of PVX was higher with antiserum having pH 8.0 and the highest when extraction buffer pH was 7.2. On the other hand, trapping of PVS was higher with antiserum having pH 6.0 and the highest with combination of antiserum pH 6.0 and extraction buffer pH either 6.0 or 8.0. Trapping of PVY also depended on the host species. Crude virus extract from potato cv. Kufri Chandramukhi was maximal with a combination of antiserum pH either 7.0 or 8.0 and extraction buffer pH 7.2, while the best combination in case of *N. tabacum* cv. Havana was the antiserum pH 6.0 or 8.0 and the extraction buffer pH 7.2. On the contrary, the best combination in the case of PVY infected *Datura metel* was the antiserum pH 7.0 or 8.0 and the extraction buffer pH 6.0. The ratio of maximal to minimal trapping varied from about 3 to 10 depending on the virus, the pH of the extraction buffer and that of the trapping antiserum.

Effect of dilutions and combinations of antisera

Data on the effect of antisera dilutions and their combinations on trapping are illustrated (Fig. 2). Maximal trapping in the case of PVX and PVY was observed with 1:1000 dilution and only slightly lower with antisera diluted to 1:600 and 1:1600. In the case of PVS, it was maximal with 1:600 but markedly reduced at 1:1600 dilution. Trapping of PVX and PVY was satisfactory with a mixture of PVX+PVY antisera at all the dilutions tested but reduced greatly with a mixture of PVX+PVS+PVY, or PVX+PVS, or PVY+PVS antisera. In other words,

Table 1. Effect of extraction buffer on trapping of potato viruses in IEM

Buffer	Average number of virus particles on the screen		
	PVX	PVS	PVY
PB	23.5 ± 2.5	7.6 ± 1.4	Aggregates
PB+EDTA	22.5 ± 2.2	3.3 ± 0.5	10.2 ± 1.8

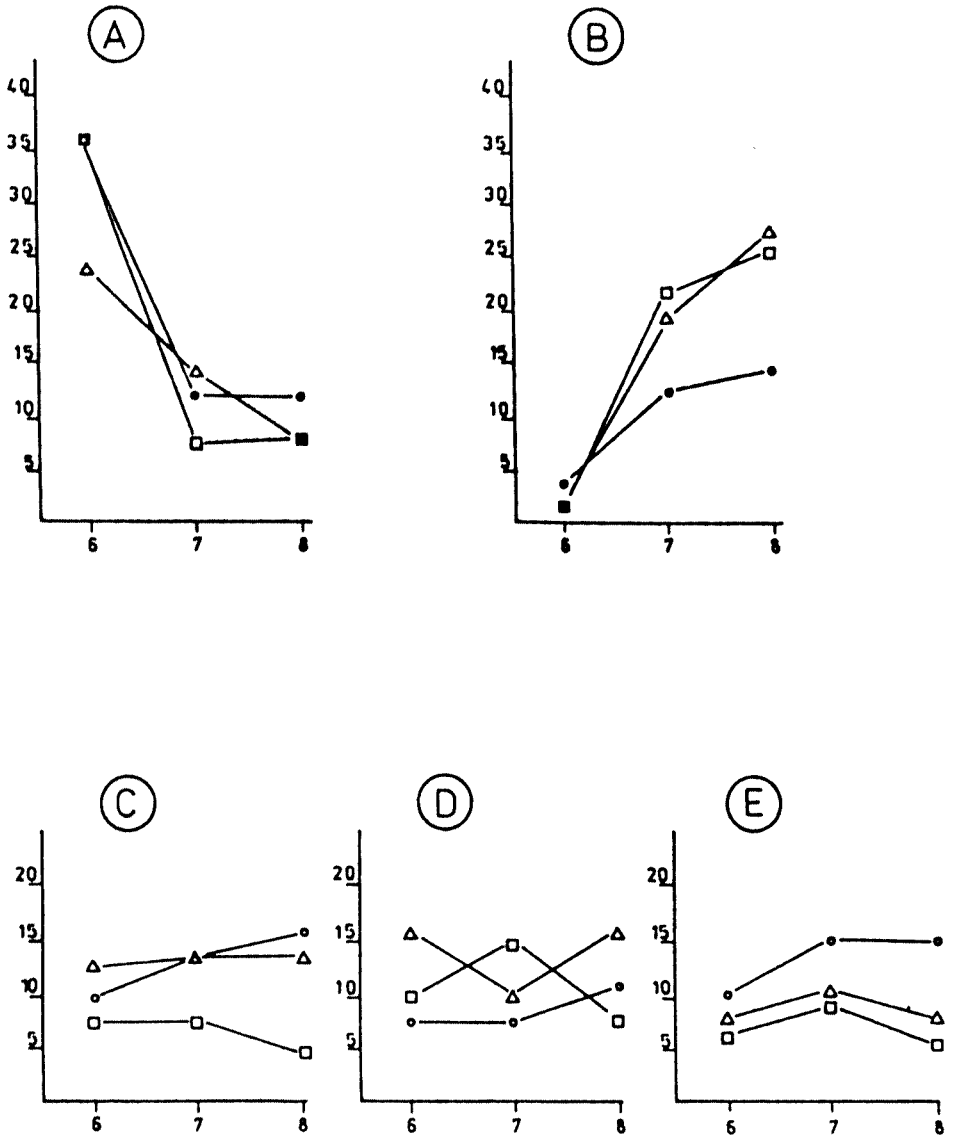

Fig. 1

Effect of host, pH of extraction buffer and antiserum on trapping of potato viruses PVS (A), PVX (B), PVY (C, D, E). Source of PVY: Potato (C), *Nicotiana tabacum* cv. Havana (D), *Datura metel* (E). pH of extraction buffer 6.0 (○), 7.2 (△) and 8.0 (□).
Ordinate: average number of trapped virions. Abscissa: pH of antiserum.

mixing of PVS antiserum with that against PVX or PVY adversely affected the trapping of PVX and PVY. Similarly, trapping of PVS was also reduced upon mixing its antiserum with either PVX or PVY or both.

Effect of simultaneous trapping

Results of studies on simultaneous trapping of more than one virus with a combination of antisera are given in Fig. 3. Simultaneous trapping resulted in reduced number of virions i. e. trapping of two or three viruses from a mixture resulted in less than half the number of virions which could have been trapped by individual antiserum. Further, when a virus trapped with individual homologous antiserum was decorated, about 50 % of the virions got dislodged during decoration. Simultaneous trapping followed by decoration also revealed that trapping of PVS and PVY together resulted in disproportionately higher amount of PVS even though the mixture contained slightly higher concentration of PVY.

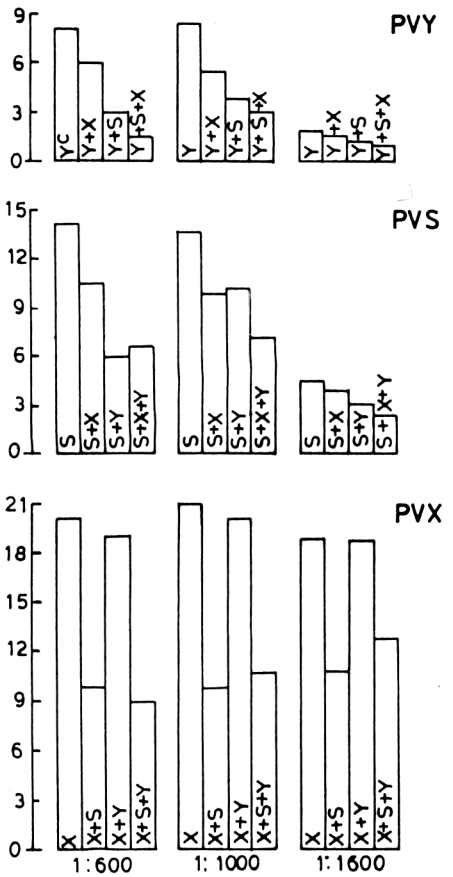

Fig. 2
Effect of combination of antisera on trapping of potato viruses
Ordinate: average number of virions trapped with antisera (columns). Abscissa: dilution of antisera.
Initial titer of all antisera was adjusted to 1:512 before preparation of the indicated dilutions.

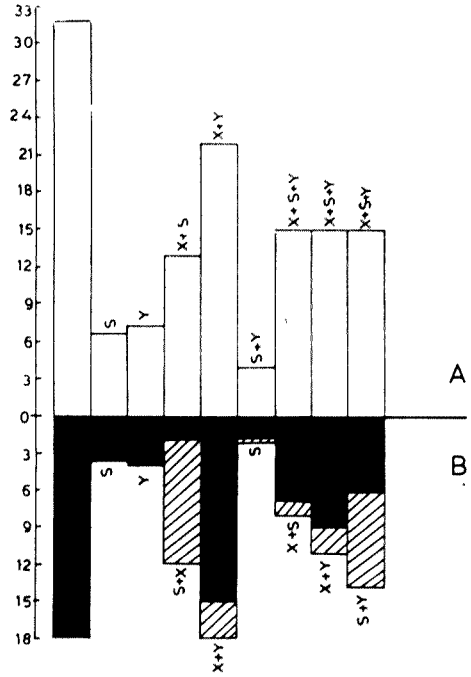

Fig. 3
Simultaneous trapping of potato viruses in IEM with mixtures of antisera
A: trapping antisera, B: decorating antisera
Virions decorated (black columns). Virions left undecorated (hatched columns).
Ordinate: average number of virions trapped. Abscissa: antisera (mixtures).

Effect of VAT

VAT had a marked effect on trapping of virions (Table 2). Per cent increase in trapping with a VAT of 6 hr as compared to 1 hr was 49–79 (for PVS) and 186–194 (for PVY), while with 24 hr VAT it was 193–198 (for PVS) and 800 (for PVY). An increase in trapping of PVX virions with increase in VAT was higher than that observed in PVS but lower than that in PVY.

Table 2. Effect of VAT on trapping of potato viruses in IEM

Antiserum dilution	Increase in trapping in %					
	PVX		PVS		PVY	
	6 hr	24 hr	6 hr	24 hr	6 hr	24 hr
1:600	110	280	79	198	194	800
1:1000	110	280	49	193	186	560

100 % = trapping at VAT 1 hr

Discussion

Trapping efficiency of the three flexuous potato viruses, PVX, PVS and PVY, differed with the extraction buffer, antiserum dilution, combinations of antisera and the VAT. Addition of EDTA to the PB for extraction of the viruses did not reduce trapping of PVX and PVY but did so in the case of PVS. EDTA may interfere with the release of PVS virions from host cells during extraction, or with the binding of virions to antibodies on the grid.

Dilutions of antisera also influenced the trapping efficiency which was observed to differ for different potato viruses. Since homologous titers of the antisera used were brought to the same level, it might be due to different amounts of antibodies required for optimal trapping of different viruses depending on their intrinsic affinity (de San Roman *et al.*, 1988). Thus, optimal trapping in the case of PVX and PVY was at 1:1000 diluted antiserum but in the case of PVS it was at 1:600. The increase in VAT from 1 to 6 and 24 hr, progressively increased the number of virions trapped. Such an increase in trapping with increase in VAT has also been reported (Cohen *et al.*, 1982; Pares and Whitecross, 1985). Increase in VAT may be particularly useful when virus titer in the extract is very low.

The level of trapping varied according to the virus, pH of the trapping antiserum and the extraction buffer, and the host species. Trapping in the case of PVY was also affected by the host species as reported earlier by Cohen *et al.* (1982).

The detection of any of the suspected viruses with a combination of antisera is advantageous for saving time and labour as reported in ELISA (Banttari and Franc, 1982; Salazar, 1979), provided it does not affect the sensitivity of IEM. The results revealed that coating with PVX+PVY antisera trapped PVX or PVY with a comparatively less adverse effect than with mixture of antisera including PVS, which trapped lesser virions. This effect could be probably due to the competition amongst different antibodies for the sites on the grids. However, a greater adverse effect of PVS antibodies could not be explained.

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References

van Balen, E. (1982): The effect of pretreatments of carbon coated formvar films on the trapping of potato leafroll virus particles using immuno specific electron microscopy. *Neth. J. Pl. Pathol.* **88.** 33–37.

Banttari, E. E., and Franc, G. D. (1982): ELISA with single and combined antisera for viruses S and X in potato tubers and plants. *Am. Potato J.* **59**, 375–383.

Braun, A. L., and Opgenorth, D. . (1987): Comparative detectability of potato leafroll virus, potato virus X and potato virus S using enzyme-linked immunosorbent assay (ELISA) and serologically

specific electron microscopy (SSEM). *Am. Potato J.* **64**, 205–207.
Cohen, J., Loebenstein, G., and Milne, R. G. (1982): Effect of pH and other conditions on immunosorbent electron microscopy of several plant viruses. *J. Virol. Methods* **4**, 323–330.
De San Roman, C. P., Logorburu, F. J., Pascualena, J., and Gil, A. (1988): Simultaneous detection of potato viruses Y, leafroll, X and S by das-ELISA technique with artificial polyvalent antibodies (APA). *Potato Research* **31**, 151–158.
Garg, I. D., and Khurana, S. M. Paul (1988): Sensitivity of immunoelectron microscopy for the detection and diagnosis of potato viruses. *Proc. 2nd Int. Symp. EM and Biophys.*, Panjab University, Chandigarh, India, pp. 275–278.
Khurana, S. M. Paul, and Garg, I. D. (1989): Plant virus, viroid and mycoplasma like organisms' detection for quarantine. *Rev. Tropical Pl. Pathol.* **6**, 251–277.
Khurana, S. M. Paul, Singh, M. N., and Shiv Kumar (1987): Purification of potato virus Y and the production of antiserum. *Curr. Sci.* **56**, 420–422.
Khurana, S. M. Paul, Singh, M. N., Garg, I. D., and Shiv Kumar (1990): Uniform purification scheme for potato viruses. *Asian Potato J.* **1**, 46–48.
Milne, R. G. (1986): New developments in electron microscope serology and their possible applications, pp. 179–191. In K. Maramorosch and H. Koprowski (Eds): *Methods in Virology VI*. Academic Press, New York.
Pares, R. D., and Whitecross, M. I. (1985): An evaluation of some factors important for maximising sensitivity of plant viruses detection by immuno-electron microscopy. *J. Virol. Methods* **11**, 339–346.
Roberts, I. M., and Harrison, B. D. (1979): Detection of potato leafroll and potato mop top viruses by immunosorbent electron microscopy. *Ann. Appl. Biol.* **93**, 289–297.
Salazar, L. F. (1979): Application de la tecnica serologica con conjugados enzimaticos (ELISA) para diagnosticar virus de papa. *Fitopatologia* **14**, 1–9 (in Spanish).
Shukla, D. D., and Gough, K. H. (1979): The use of protein A, from *Staphylococcus aureus*, in immune electron microscopy for detection of plant virus particles. *J. gen. Virol.* **45**, 533–536.
Singh, M. N., Khurana, S. M. Paul, and Garg, I. D. (1990): Antiserum production and immuno-electron microscopy of potato leafroll virus. *Indian Phytopathol.* **43**, 13–19.