# Detection of Mixtures of Bean and Cowpea Viruses by Using Single and Mixed Antisera in Enzyme-linked Immunosorbent Assay

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## Abstract

*The sensitivity of ELISA to detect bean common mosaic potyvirus (BCMV), cucumber mosaic cucumovirus (CMV), bean yellow mosaic potyvirus (BYMV), cowpea mottle carmovirus (CPMoV), cowpea mosaic comovirus (CPMV) and blackeye cowpea mosaic potyvirus (BLCMV) singly or in mixtures was evaluated using single or mixed antisera and the results compared to standard ELISA. The sensitivity was evaluated by comparing the absorbances ($A_{405}$) values between treatments. Significantly ($P \leq 0.05$) higher $A_{405}$ values were recorded in wells coated with mixture of antisera than in the wells coated with single antiserum. Virus mixtures tested with antisera mixtures gave significantly ($P \leq 0.05$) higher $A_{405}$ values than test involving a single virus probed with a single antiserum. The results thus indicate that two or more viruses in bean or cowpea leaf tissues or in bean + cowpea mixtures can be detected by using antisera mixtures in a single well without any loss of sensitivity. The implications are discussed in light of the ever increasing efforts by virus researchers worldwide to optimize ELISA procedures in order to reduce the time and the costs involved in carrying out the test.*

**Key words:** Antisera, Antisera mixture, Bean, Cowpea, ELISA, Sensitivity, Virus, Virus mixture.

## Introduction

Enzyme linked immunosorbent assay (ELISA) originally a medical immunodiagnostic assay, was first introduced in plant virology by Voller *et al.* (1976). Since then the method has been adapted for routine testing of plants and plant parts for the presence of viruses, bacteria, fungi and mycoplasma (Lange *et al.*, 1983). However, the standard ELISA procedure which requires the use of a single specific antiserum for a single specific virus is cumbersome, time consuming and expensive when several viruses are to be tested. For a routine use of ELISA (e.g. in seed health testing centers, in quarantine stations and in seed certification schemes), requires for the development of a simple, low cost ELISA technique which should be able to detect several viruses and which is affordable to most of the poor third world laboratories (Joshi and Albrechtsen, 1992).

Several attempts have been done to adapt ELISA for quantitative and qualitative detection of single or several viruses in a single micro ELISA plate. For example, Stobbs *et al.* (1985), Bar-Joseph *et al.* (1983) and Banttari and Peterson (1983) reported that re-washing of micro-ELISA plate did not have any significant effect on the sensitivity of ELISA. Banttari and Franc (1982) reported that a mixture of antisera was as effective as single antiserum in detecting potato virus s (PVS) and potato virus x (PVX) in potato. More recently, Joshi and Albrechtsen (1992) detected cowpea aphid-borne mosaic potyvirus (CAMV), cowpea mottle carlavirus (CPMoV), cucumber mosaic cucumovirus (CMV), southern bean mosaic sobemovirus and cowpea mosaic comovirus (CMV) in cowpea samples using a mixture of

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antisera. The use of a mixture of antisera to test for a range of viruses as a time and supplies serving measures has also been recommended by Grim and Daniel (1984) and Etiene *et al.* (1991). Despite the work done so far on the effectiveness of using antisera mixtures for detection of several viruses in plant materials; it has not been clearly proven whether a mixture of viruses in different plant tissues can be detected in a single micro ELISA well with a mixture of antisera without compromising the sensitivity of the test.

The objective of this study was to investigate the effect of using antisera and viruses mixtures on the sensitivity of ELISA to detect BCMV, CMV, BYMV, CPMoV, BLCMV and CPMV in bean and cowpea leaf samples.

# Materials and Methods

## Source of test materials

Seeds of beans and cowpea were collected from plant known to be infected with one of the following viruses: BLCMV, CPMV, CPMoV BCMV and CMV. The seeds were collected from various locations and/or research stations in Tanzania as follows: bean seeds, PL47 (Ilonga, Kilosa), PL30 (MARTI-Uyole), PL 48; S52, S53, S54, S55, S56 and S57 (Selian, Arusha) and cowpea seeds, S47 (Mikumi, Morogoro), S13, S20 and S3 (Ilonga, Kilosa). Authenticated isolates of BYMV, BLCMV, CPMV, CPMoV, BCMV and CMV in desiccated leaf tissues were received from the Danish Institute for Seed Pathology for developing countries (DGISP) via the Tanzania Official Seed Certification Agency (TOSCA) and were used as tests or as positive controls. Leaf tissues from healthy seedlings of cowpea and bean were used as negative controls.

## Planting of seed material

Forty seeds of each bean and cowpea accessions from plants previously infected with one or a mixture of BCMV, CMV, BLCMV, CPMoV and CPMV were sown in heat sterilized Forrest soil in two-liter plastic pots at the rate of five seeds per pot at TOSCA, Morogoro, Tanzania. Ten days after germination, the seedlings were transplanted into one-liter plastic pots at one plant per pot filled with sterilized Forrest soil. The pots were maintained in the greenhouse *at* 25-35°C under natural light until maturity. For BYMV, 40 healthy bean seedlings were inoculated with the virus when they were 10-days old.

## Sources and preparation of antisera

Crude polyclonal antisera against BCMV, CMV, BYMV, BLCMV, CPMoV and CPMV were diluted in serum (coating) buffer containing 20g polyvinypyrrolidone (MW=25,000), 2g skimmed milk and 0.02% $NaN_3$ pH 7.2. The sources, specifications and dilutions of polyclonal antisera used in this study are shown in (Table 1).

**Table 1. Identity, source and dilution of anti-viral antisera used in this study**

| Antisera | Source | Dilution |
|---|---|---|
| Cowpea viruses | | |
| BLCMV[1] | ATCC[7] | 1:100 |
| CPMV[2] | DGISP[8] | 1:1000 |
| CPMoV[3] | ATCC[7] | 1:2500 |
| Bean viruses | | |
| BCMV[4] | ATCC[7] | 1:1000 |
| CMV[5] | ATCC[7] | 1:1000 |
| BYMV[6] | DGISP[8] | 1:2500 |

**[1]Black eye cowpea mosaic virus, [2]Cowpea mosaic virus, [3]Cowpea mottle virus, [4]Bean common mosaic virus, [5]Cucumber mosaic virus, [6]Bean yellow mosaic virus, [7]American Type Culture Collection, USA,**
**[8]Danish Government Institute of Seed Pathology, Denmark**

## Cross absorption of primary antisera

In order to reduce the incidences of non specific reactions with homologous antigens, the Polyclonal antisera used was first cross-absorbed with the appropriate healthy cowpea or bean tissue extracts in serum buffer and incubated at 37 °C for 1h before use (Hobbs *et al.*, 1987).

## Sample preparation and plate formatting

Plants were assayed for the presence of virus (es) at the first trifoliolate leaf stage. Test sample typically consisted of 30 6-mm leaf discs (Romaine *et al.*, 1981) punched from stacks of 10 trifoliolate leaves, 3 punches per stack, each leaf representing a separate Vigna or Phaseolus seedlings. The 30 leaf discs for each sample, weighing approximately 0.6 to 1.2 g were placed into 76 x 122-mm zip – lock bag ( Cole – Parmer Instruments, Chicago, U.S.A)

with 1ml of antigen buffer (PBS + .01 M NaDIECA) and homogenized by repeated roller action applied externally with 12 x 110 test tubes. Homogenate obtained from each bag was further diluted to a total of 100- fold, in a labeled rack of tubes. One hundred-$\mu$l aliquots from this pool of prepared antigen were placed into four wells per plate, each plate tested by a single or mixtures of anti-viral antiserum. Positive controls consisted, respectively, of homogenized-infected plant tissue (1:10) in antigen buffer) diluted to 1000- fold with healthy-plant homogenate (1:100 in antigen buffer. Negative controls consisted of homogenized healthy-plant tissues diluted 100-fold in antigen buffer. The Multiskan Plus' plate reader was blanked against wells to which antigen buffer had been applied in place of plant sap (Hampton *et al.*, 1992).

## Serology

The ELISA was used as described by Hobbs *et al.* (1987) with slight modification as follows. In tests to detect samples for one or several viruses using single or mixed antisera, about one hundred microlitre of each sample was loaded into each well of microtitre plate in four replications. Negative and positive controls also were loaded separately in four replications. Half of the bean and cowpea test samples were exposed to their specific crossed absorbed antisera and the other half were exposed to the mixture of antisera. After one hour incubation, the plates were washed three times with phosphate buffered saline tween (PBST) buffer, with an interval of three minutes between wash. The plates were incubated with 1:1000 diluted anti-rabbit IgG produced in swine and conjugated to alkaline phosphate at 37 °C for 1h. The plates were washed again as described above and then incubated with the substrate Para-nitrophenyl phosphate ($P_NPP$) for 30 minutes. The color intensity was measured by using ELISA reader as absorbance at 405nm ($A_{405}$). In tests to determine whether bean and cowpea samples with multiple viral infection can be detected with mixture of antisera, similar procedure as described above was adopted using mixture of antigens (5ml of each of cowpea antigens and 5ml of each of bean antigens) mixed in all possible combinations of the viruses (Table 4 and 5). Threshold values for positive reactions was calculated from an average of $A_{405}$ readings of corresponding healthy wells plus 5 times the standard deviation among test wells as described by Sutula *et al.* (1986).

# Results and discussion

The $A_{405}$ values for the different treatments are presented in Tables 2, 3, 4 and 5. In tests involving the use of mixture of anti-viral antisera against individual virus in bean, the $A_{405}$ values were significantly ($P \leq 0.05$) higher in the tests involving anti-viral antisera mixtures than in the tests involving a single anti-viral antiserum (Table 2). This suggests that bean leaf singly infected with one of the viruses under test can be detected in a single micro-ELISA well by using a mixture of the three anti-viral antisera without affecting the sensitivity of the test. Similar results were obtained with cowpea samples (Table3). The $A_{405}$ values of the mixture of anti-viral antisera against BLCMV or CPMoV or CPMV in cowpea were significantly ($P \leq 0.05$) higher than in tests involving a single anti-viral antiserum. These results are in agreement with the work of Joshi and Albrechtsen (1992) Bantari and Franc (1982) Etienne *et al.* (1991) and Grimm and Daniel (1984) who reported higher $A_{405}$ values in tests involving antisera mixtures for cowpea, grapevine and potato viruses respectively.

**Table 2. Absorbance values (average of four wells) showing the sensitivity of detection by ELISA of bean viruses using anti-viral antiserum separately or in mixture [1]**

| Sample accession no.[2] | BCMV[3] | CMV[4] | BYMV[5] | BCMV+CMV+BYMV |
|---|---|---|---|---|
| PL-80 (BYMV) | $0.11^{kl}$ | $0.20^{ghi}$ | $0.21^{ghi}$ | $0.73^{b}$ |
| PL-47 ( CMV) | $0.19^{ghi}$ | $0.22^{g}$ | $0.19^{ghi}$ | 0.62c |
| PL-48 (BYMV) | $0.22^{g}$ | $0.20^{ghi}$ | $0.59^{cd}$ | $0.82^{a}$ |
| S-52 (BYMV) | $0.21^{gh}$ | 0.19ghi | $0.73^{b}$ | $0.69^{b}$ |
| S-53 ( BCMV) | $0.19^{ghi}$ | $0.21^{ghi}$ | $0.21^{ghi}$ | $0.61^{c}$ |
| S-54 (BYMV) | $0.21^{ghi}$ | $0.19^{ghi}$ | $0.31^{f}$ | $0.56^{cd}$ |

| | | | | |
|---|---|---|---|---|
| S-55 (CMV) | $0.11^{kl}$ | $0.29^{f}$ | $0.17^{ghijk}$ | $0.41^{e}$ |
| S-56 (BCMV) | $0.19^{ghi}$ | $0.19^{ghi}$ | $0.18^{ghij}$ | $0.54^{d}$ |
| S-57 (CMV) | $0.12^{jkl}$ | $0.39^{e}$ | $0.15^{ijkl}$ | $0.71^{b}$ |
| Negative control | $0.11^{l}$ | $0.11^{l}$ | $0.11^{l}$ | $0.11^{l}$ |
| Positive control | $0.70^{b}$ | $0.70^{b}$ | $0.70^{b}$ | $0.70^{b}$ |
| LSD = 0.06 | | | | |
| SE± 0.19 | | | | |

**[1]Threshold ($A_{405}$) value=0.12, [2]Multiply infected with BCMV + CMV + BYMV. [3]Bean common mosaic virus, [4]Cucumber mosaic virus, [5]Bean yellow mosaic virus.**
**Mean followed by the same letter do not differ significantly (P≤0.05) according to DMRT.**

**Table 3. Absorbance values (average of four wells) showing the sensitivity of detection by ELISA of cowpea viruses using anti-viral antiserum separately or in mixture [1]**

| Sample accession no.[1] | BLCMV3 | CPMoV4 | CPMV[5] | BLCMV+CPMoV+CPMV |
|---|---|---|---|---|
| S-47 (BLCMV) | $0.16^{h}$ | $0.45^{def}$ | $0.43^{def}$ | $0.36^{ef}$ |
| S-20 (CPoMV) | $0.14^{h}$ | $0.21^{h}$ | $0.51^{bcd}$ | $0.34^{fg}$ |
| 13-20 (BLCMV) | $0.36^{e\,f}$ | $0.11^{h}$ | $0.62^{ab}$ | $0.53^{bcd}$ |
| S-3 (CPMV) | $0.12^{h}$ | $0.12^{h}$ | $0.22^{gh}$ | $0.61bc^{c}$ |
| Negative control | $0.12^{h}$ | $0.12^{h}$ | $0.12^{h}$ | $0.12^{h}$ |
| Positive control | $0.74^{a}$ | $0.74^{a}$ | $0.74^{a}$ | $0.7a^{b}$ |
| LSD = 0.13 | | | | |
| SE± 0.04 | | | | |

**[1]Threshold ($A_{405}$) value=0.16, [2]Multiply infected with BLCMV, CPMV and CPMoV, [3]Blackeye cowpea mosaic virus, [4]Cowpea Mottle Virus, 5Cowpea mosaic virus**
**Mean followed by the same letter do not differ significantly (P≤0.05) according to Duncan Multiple Range Test**

In tests involving simultaneous detection of bean viruses in a pooled sample containing more than one virus using anti-viral antisera mixtures, the $A_{405}$ values of virus mixtures probed with mixtures of anti-viral antiserum were significantly($P \leq 0.05$) higher than tests involving probing a sample with a single anti-viral antiserum (Table4). Similarly, in cowpea samples doubly infected with [(BLCMV + CPMV) or (CPMV + CPMoV) or (BLCMV + CPMoV)] or multiply infected with (BLCMV + CPMV +CPMoV), the $A_{405}$ values were significantly ($P \leq 0.05$) higher in tests involving the probing of the virus mixtures with anti-viral antisera mixtures than tests involving the detection of individual viruses using single anti-viral antiserum (Table5). The results suggest that mixing of viruses from bean and cowpea leaf tissues does not affect the sensitivity of ELISA. In some tests the A405 values of virus mixtures probed with antisera mixtures was doubled indicating an additive effect (Table 2, 3 and 4).

Until now the ELISA has been used for large-scale programmes, where many seed samples and many seed per sample are tested for the presence of the same virus. Immunosorbent Electron Microscope (ISEM) is the only technique used when screening for several viruses. However, ISEM is very expensive, highly sophisticated and not available in the third would countries (Lange *et al.,* 1983). Development of a highly sensitive ELISA technique suitable for testing several viruses in a single micro-ELISA well, would be highly welcomed in the third world countries' laboratories where funding and reagents is the most limiting. However, for any procedure to be of use in plant and seed health testing it must include the following three aspects: 1) Adaptation to detect the viruses in large quantities of plant and plant parts. 2) High sensitivity is needed as some viruses occur in lower percentages especially in plants. 3) The procedure should be simple and suitable for large scale routine progrmmes in laboratories which are not equipped for sophisticated virology (Lange *et al.,* 1983). Our technique, at least in this study, meets all the three criteria.

**Table 4: Absorbance values (average of four wells) showing sensitivity of detection by ELISA of mixtures of bean viruses using anti-viral antiserum separately or in mixture [1]**

| Virus Mixtures | Anti-viral antiserum BCMV[2] | CMV[3] | BYMV[4] | BCMV+CMV+BYMV |
|---|---|---|---|---|
| BCMV+CMV+BYMV | 0.26$^{f}$ | 0.51$^{cde}$ | 0.16$^{fg}$ | 0.64$^{b}$ |
| BCMV+CMV | 0.11$^{g}$ | 0.45$^{de}$ | (-) | 0.87$^{a}$ |
| CMV+BYMV | (-) | 0.60$^{bc}$ | 0.4$^{0e}$ | 0.88$^{a}$ |
| BCMV+BYMV | 0.09$^{g}$ | (-) | 0.11$^{g}$ | 0.94$^{a}$ |
| BCMV | 0.64$^{b}$ | (-) | (-) | 0.51$^{cde}$ |
| CMV | (-) | 0.93$^{a}$ | (-) | 0.44$^{de}$ |
| BYMV | (-) | 0.83$^{a}$ | 0.13$^{g}$ | 0.60fbc$^{g}$ |
| Negative control | 0.08$^{g}$ | 0.08$^{g}$ | 0.08$^{g}$ | 0.08$^{g}$ |
| LSD = 0.12 | | | | |
| SE± = 0.06 | | | | |

**[1]Threshold value ($A_{405}$)= 0.15, [2]Bean common mosaic virus, [3]Cucumber mosaic virus, [4]Bean yellow mosaic virus, (-) not tested.**
**Means followed by the same letter do not differ significantly (P≤0.05) according to Duncan Multiple Range Test (DMRT).**

**Table 5. Absorbance values (average of four wells) showing sensitivity of detection by ELISA of mixture of cowpea antigens using anti-viral antiserum separately or in mixture [1]**

| Mixture of sample Antigens | CPMV[2] | CPMoV[3] | BLCMV[4] | CPMV+CPMoV+BLCMV |
|---|---|---|---|---|
| BLCMV+CPMV+CPMoV | 0.42$^{k}$ | 0.54$^{1}$ | 0.44$^{jk}$ | 0.51$^{ijk}$ |
| BLCMV+CPMV | 0.56$^{ghi}$ | (-) | 0.49$^{ijk}$ | 0.52$^{ij}$ |
| CPMV+CPMoV | 0.57fghi | 0.79$^{abc}$ | (-) | 0.84$^{a}$ |
| BLCMV+CPMoV | (-) | 0.19$^{lm}$ | 0.10$^{m}$ | 0.11$^{m}$ |
| BLCMV | (-) | (-) | 0.25$^{l}$ | 0.43$^{k}$ |
| CPMV | 0.52$^{ij}$ | (-) | (-) | 0.54$^{i}$ |
| CPMoV | (-) | 0.81$^{ab}$ | (-) | 0.79$^{abc}$ |
| Negative control | 0.12$^{m}$ | 0.12$^{m}$ | 0.12$^{m}$ | 0.12$^{m}$ |
| LSD = 0.09 | | | | |
| SE± = 0.05 | | | | |

**[1]Threshold value ($A_{405}$)=0.13, [2]Cowpea mosaic virus, [3]Cowpea mottle virus, [4]Blackeye cowpea mosaic virus, [5](-) not tested.**
**Means followed by the same letter do not differ significantly (P≤0.05) according to Duncan Multiple Range Test (DMRT).**

One of the many advantages of using antisera mixtures in virus testing is that one does not have to probe the sample with different antiserum each time a specific virus is to be tested. This procedure, therefore, is very useful where a large number of samples are to be screened for several viruses whereby time and reagents are limiting. The conventional ELISA procedure of testing one virus at time is time consuming and may not always needed where the purpose is to test for freedom from a range of viruses and where the identification of individual viruses is not required (Joshi and Albrechtsen, 1992).

## Conclusion

The use of mixed antisera has the potential of being very rapid and reliable detection method for routine seed and plant health testing. This technique saves the time and reduces test consumables because several viruses can be detected in a single well. Also, mixing of viruses from different plant materials of the same genus does not affect the sensitivity of the ELISA.

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## References

Banttari, E. E. and Franc, G. D. (1982). Enzyme linked immunosorbent assay with single orcombined antisera for viruses S and X in potato tubers and plants. American Potato Journal 52:375-387.

Banttari, E. E and Peterson, A., (1983). A cleaning technique for cuvettes used in enzyme-linked immunosorbent assay. Plant Disease. 67:18-20

Bar-Joseph, M., Muscovitz, M., and Sharafi, Y. (1983). Re-use of coated enzyme-linkedimmunosorbent assay plates. Phytopathology. 69:424-426.

Etienne, L., Clauzel, J. M. and Fuchs, M. (1991). Simultaneous detection of several nepoviruses infecting grapevine in a single DAS-ELISA test using mixed antisera.Phytopathology. 131:89-100.

Grimm, F. and Daniel, G. (1984). On the use of mixed sera with ELISA: a comparison of results with single antisera in studies with potato leaves. Potato Research. 27: 13-23.

Hampton, R. O., Albrechtsen, S. E. and Mathur, S.B. (1992). Seed healthy (viruses) of *Vigna unguiculata* selections from developing countries Seed Sci. & Technol., 20: 23-38.

Hobbs, H. A., Reddy, D. V.R., Rajeshwari, R., and Reddy, A. S. (1987). Use of direct antigen coating and protein A coating ELISA procedures for detection of three peanut viruses. Plant Disease 71:747-749.

Joshi, S. and Albrechtsen. S. E. (1992). Use of mixed and individual antisera in ELISA detection of viruses infecting cowpea and soybean. Journal Institute of Agriculture and Animal Science. 13: 83-87.

Lange, L., Tien, P. and Begtrup, J. (1983). The potential of ELISA and ISEM in seed health tesing. Seed Sci. and Technol. 11,477 -490.

Romaine, C. P., Newhart, S. R., and Anzalo, D. (1981). Enzyme linked immunosorbent assay for viruses in intact leaf tissue disks. Phytopathology 71, 308-312.

Sutula, C. L., Gillete, J. M., Morrissey, S. M. and Ramsdell, D. C. (1986). Interpreting ELISA data and establishing the positive-negative threshold. Plant Disease. 70: 722-726.

Stobbs, Z. W. and Van Schagen J. G. (1985). Reconditioning of ELISA plates. Phytopathology 76: 483-486.

Voller, A., Bidwell, D. E., Clark, M. F. and Adams, A. N. (1976). The detection of viruses by Enzyme-linked immunosorbent assay (ELISA). Journal of General Virology 33: 165-67.