# Comparison of three different tests for detection of cucumber mosaic cucumovirus in banana (*Musa paradisiaca*)

**G. Kiranmai, P. Sreenivasulu and M. V. Nayudu**
Department of Virology, S. V. University, Tirupati 517 502, India

**Direct ELISAs like double antibody sandwich (DAS)–ELISA, simplified rapid direct antigen coating (SRDAC)–ELISA and indirect form of DAC-ELISA for detection of cucumber mosaic cucumovirus (CMV) causing infectious chlorosis of banana in leaf extracts and pseudostem sap exudates and purified virus diluted with antigen buffer or healthy banana leaf sap were compared. The sensitivity levels of three ELISA tests were similar and the virus was detected up to $10^{-7}$ dilution with leaf extract, 1 μl/well with sap exudate, 20 ng/well with purified CMV and 100 ng/well with purified virus diluted with healthy banana leaf extract. Of three forms of ELISA compared, DAC-ELISA was further evaluated with detection of infectious virus by local lesion bioassay on cowpea (*Vigna unguiculata*) and by double stranded RNA (dsRNA) analysis. The banana leaf and pseudostem sap exudate samples that were positive in DAC-ELISA were also positive by other two tests. Collection of pseudostem sap exudates by pin pricking from test plants and detection of virus by DAC-ELISA in them appears ideal for large-scale testing of banana plants.**

THE banana (*Musa paradisiaca*) is one of the world's most important tropical fruit crops. It is grown in all types of tropical agricultural systems[1]. Bananas are propagated vegetatively through suckers. Viruses as systemic pathogens are readily disseminated in vegetative propagation material and usually cause serious problems in crops propagated in this way.

The causal viruses of bunchy top (banana bunchy top virus-BBTV), infectious chlorosis (cucumber mosaic cucumovirus-CMV), bract mosaic (banana bract mosaic potyvirus-BBMV), streak (banana streak badnavirus-BSV) and tobacco mosaic virus[1–4] diseases of banana are vertically transmitted through planting suckers.

Four viruses are known to naturally infect banana in East and West Godavari districts of Andhra Pradesh state in India (personal communication, Krishna Prasadji, Banana Research Station, APAU, Kovvur). The field identification of diseases caused by CMV, BBMV and BSV based on the symptoms in banana is difficult as they induce similar symptoms (chlorotic streaks) at certain stages of disease development.

The infectious chlorosis disease caused by CMV has been reported from India, Australia, Greece and Moracco[5–14]. Recently, a virus causing severe chlorosis/mosaic disease of banana in Uttar Pradesh, India was identified as a strain of CMV using slot-blot hybridization with nucleic acid probe of CMV-P genome[15]. Currently, the recommended strategy for control of CMV in banana is to identify virus-infected plants, remove the diseased plants and replant with virus-free banana plants[1]. In order for this approach to be successful, it is necessary to have sensitive, rapid and reliable indexing tests for the detection of CMV from banana plants. Hansen and Wick[16] reviewed the various biological, immunological and biochemical tests used for detection and identification of plant viruses. Of these, ELISA-based tests have been widely used because of their simplicity, economy and adaptability for large-scale testing of suspected plant samples.

In view of potential importance of CMV in banana[17], we evaluated three ELISA procedures and compared one of them with bioassay and double stranded (ds) RNA analysis for detection of CMV in banana leaf extracts and pseudostem sap exudates collected by pin pricking.

CMV causing infectious chlorosis in banana plants growing under natural conditions in Andhra Pradesh served as the source of primary inoculum. The virus culture was initially established on cowpea and single lesion isolate was subsequently propagated on tobacco by sap inoculation and on banana by injecting the purified virus[18]. Virus-free banana plants were propagated by transplanting virus-free suckers indexed by DAC-ELISA. The virus was purified from virus-infected tobacco leaves harvested twenty days after sap inoculation essentially by following the procedure of Walkey[19]. The concentration of purified virus was determined by considering 5 OD units = 1 mg (ref. 20).

Polyclonal antiserum prepared previously to CMV-banana isolate was used in this study[18]. Immunoglobulins (IgG) from crude CMV-banana antiserum for direct ELISA procedures were purified by sodium sulphate precipitation followed by dialysis as described by Rajeswari *et al.*[21]. Alkaline phosphatase (Sigma) was conjugated to CMV IgG and goat antirabbit antibodies (Gibco, USA) by one step glutaraldehyde method[22].

The composition of buffers and plate-washing procedures were similar to those of Clark and Adams[22]. The volumes of reactants added to each step unless otherwise stated were 200 μl/well. All ELISA incubation periods prior to substrate addition were 90 min at 37°C in a humid box. ELISA plates with flat bottom wells (Laxbro, Pune) were used in all procedures.

The antigen samples for simultaneous testing used were banana pseudostem sap exudate collected separately from healthy and CMV-infected banana by pin pricking the pseudostem (10, 20, 50, 100 μl/ml), banana leaf sap ($10^{-1}$–$10^{-8}$ dilution), purified CMV-banana (0.1–2.0 μg/ml). For direct antigen coating procedures (DAC, SRDAC) the antigens were extracted (1 g/9 ml buffer) and diluted in 0.05 m carbonate buffer, pH 9.6

**Table 1.** Detection of CMV causing infectious chlorosis of banana by different forms of ELISA

| Type of antigen | Antigen concentration[a] | Direct form | | Indirect form |
|---|---|---|---|---|
| | | DAS-ELISA[b] | SRDAC-ELISA[c] | DAC-ELISA[d] |
| CMV-infected banana leaf sap | $10^{-1}$ | 2.80[e] | 1.93 | 3.0 |
| | $10^{-2}$ | 2.62 | 1.85 | 3.0 |
| | $10^{-3}$ | 2.02 | 1.53 | 2.20 |
| | $10^{-4}$ | 1.18 | 1.12 | 2.01 |
| | $10^{-5}$ | 0.94 | 0.85 | 1.13 |
| | $10^{-6}$ | 0.82 | 0.73 | 1.00 |
| | $10^{-7}$ | 0.31 | 0.28 | 0.33 |
| | $10^{-8}$ | 0.09 | 0.07 | 0.10 |
| Healthy banana leaf sap | $10^{-1}$ | 0.06 | 0.07 | 0.05 |
| | $10^{-2}$ | 0.02 | 0.03 | 0.03 |
| CMV-infected banana pseudostem sap exudate (µl/ml) | 100 | 2.84 | 2.20 | 3.01 |
| | 50 | 2.71 | 1.91 | 3.0 |
| | 20 | 2.04 | 1.50 | 2.32 |
| | 10 | 1.25 | 0.94 | 1.52 |
| | 5 | 0.41 | 0.23 | 0.43 |
| | 2 | 0.04 | 0.05 | 0.02 |
| Healthy pseudostem sap exudate (µl/ml) | 100 | 0.09 | 0.10 | 0.07 |
| | 50 | 0.08 | 0.05 | 0.06 |
| Purified CMV (µg/ml) diluted with antigen extraction buffer | 2.0 | 2.53 | 2.71 | 2.75 |
| | 1.0 | 2.09 | 1.98 | 2.14 |
| | 0.5 | 1.14 | 1.03 | 1.20 |
| | 0.1 | 0.51 | 0.87 | 0.40 |
| | 0.01 | 0.04 | 0.03 | 0.06 |
| Purified CMV diluted with healthy banana leaf sap (µg/ml) extracted with antigen extraction buffer | 2.0 | 0.97 | 0.95 | 1.21 |
| | 1.0 | 0.63 | 0.70 | 1.13 |
| | 0.5 | 0.32 | 0.42 | 0.64 |
| | 0.1 | 0.04 | 0.02 | 0.07 |

[a]Antigens (Sap, purified virus and pseudostem sap exudate) diluted in PBS–TO containing DIECA for DAS-ELISA and in carbonate buffer containing DIECA for DAC and SRDAC-ELISA; [b,c]ALP labelled CMV-B antibodies used at 1:500; [d]ALP labelled goat antirabbit antibodies used at 1:1000; [e]Values are an average absorbance $A_{405}$ of three wells recorded after 90 min of adding substrate.

containing 0.01 M DIECA. Whereas for DAS-ELISA they were extracted and diluted in PBS-TO containing 0.01 M DIECA. The antigen dilutions in each procedure were replicated in three wells in each of the experiments.

Antiserum and enzyme-conjugated IgG were diluted in PBS-TO. *p*-Nitrophenyl phosphate (PNP) prepared just before use at 0.5 mg/ml diethanolamine buffer, pH 9.8 was used as a substrate, NaOH (3M) at 50 µl/well used to terminate the reactions.

DAS-ELISA described by Clark and Barjoseph[23] was followed. CMV-banana IgG at 1:500 in carbonate buffer, pH 9.6 was added to the plates as trapping antibodies. Antigen samples prepared in PBS-TO were added after washing the plate, followed by alkaline phosphatase labelled CMV-IgG at 1:500 dilution. After washing, the substrate was added and incubated at room temperature for 90 min. Absorbance readings ($A_{405}$) were recorded with Bio-Tek Ceres 900 ELISA reader over buffer controls. Readings twice those of healthy were considered as positive.

The procedure for SRDAC-ELISA was similar to DAS, but here the plates were directly coated with antigen samples prepared in carbonate buffer, pH 9.6 (ref. 24). The trapped antigens were detected by alkaline phosphatase labelled CMV-IgG as described above.

For DAC-ELISA, the plates were directly coated with antigen samples prepared in carbonate buffer, pH 9.6 (ref. 25). Crude CMV-banana polyclonal antiserum at 1:500 dilution in PBS-TO was added after washing. Alkaline phosphatase labelled goat antirabbit antibodies at 1:1000 in PBS-TO were added to the plate to detect the antigen–antibody reaction. The plate was incubated with *p*-nitrophenyl phosphate for 90 min at room temperature. The absorbance values were recorded as described under DAS-ELISA.

The virus-infected banana leaf samples from different plants were separately ground (1 g/9 ml) in inoculation buffer (0.01 M $KPO_4$ buffer, pH 7.2 containing 0.2% 2-mercaptoethanol) and sap inoculated to carborundum powder (600 mesh) dusted *Vigna unguiculata* plants and observed for development of local lesions. Pseudostem

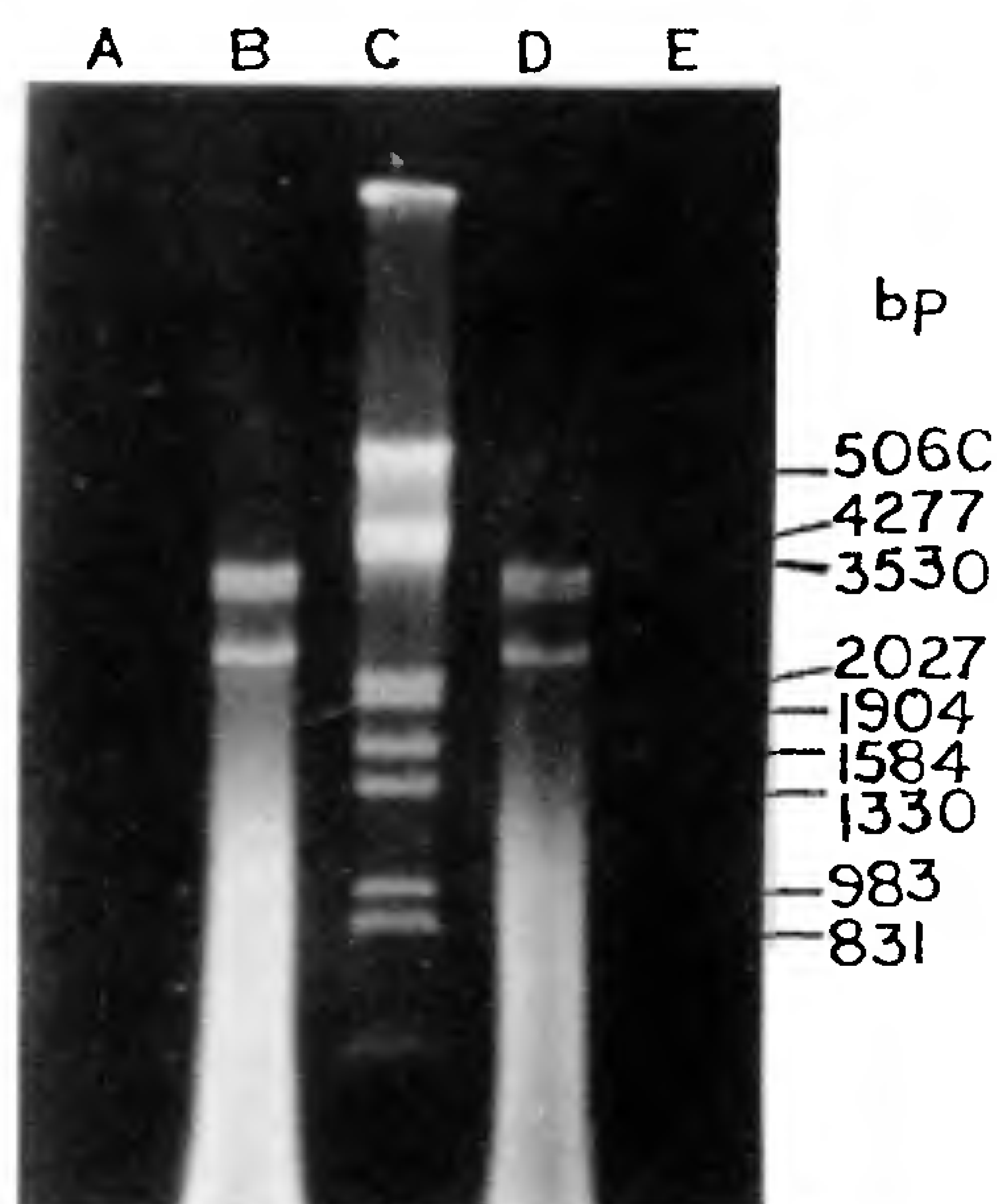


**Figure 1.** dsRNA analysis of CMV–Banana isolate in 2% agarose gel. Lane A, dsRNA isolated from healthy banana pseudostem sap exudate; B, dsRNA isolated from CMV-infected banana pseudostem sap exudate; C, λDNA/*Eco*RI & *Hin*dIII digested marker (Mr values are shown on right side); D, dsRNA isolated from CMV-infected banana leaf sap; E, dsRNA isolated from healthy banana leaf sap.

**Table 2.** Evaluation of DAC-ELISA for detection of CMV in banana leaves and pseudostem sap exudate

| Nature of banana plant | Leaves | Pseudostem sap sap exudate |
|---|---|---|
| CMV-infected banana | | |
| 1 | 3.02[a] | 3.55 |
| 2 | 2.97 | 3.48 |
| 3 | 3.11 | 3.31 |
| 4 | 3.17 | 3.27 |
| 5 | 3.01 | 3.52 |
| 6 | 2.92 | 3.47 |
| 7 | 2.85 | 3.25 |
| 8 | 3.07 | 3.29 |
| 9 | 3.05 | 3.40 |
| 10 | 2.90 | 3.38 |
| 11 | 3.01 | 3.37 |
| 12 | 3.0 | 3.19 |
| Healthy banana | | |
| 1 | 0.07 | 0.05 |
| 2 | 0.07 | 0.02 |
| 3 | 0.03 | 0.07 |
| 4 | 0.10 | 0.11 |
| 5 | 0.08 | 0.09 |
| 6 | 0.12 | 0.08 |
| 7 | 0.04 | 0.07 |
| 8 | 0.11 | 0.03 |
| 9 | 0.09 | 0.07 |
| 10 | 0.05 | 0.04 |

[a]Figures represent an average of three $A_{405}$ readings.

sap exudate was separately collected by pin pricking from different infected plants and diluted with inoculation buffer (500 μl/ml). This sap was inoculated to abrasive dusted *V. unguiculata* plants.

The dsRNA was isolated from CMV-infected banana leaves and pseudostem sap exudates by lithium chloride fractionation method of Diaz-Ruiz and Kaper[26] and analysed by 2% agarose gel electrophoresis[27].

The concentrations of crude antiserum, IgG, enzyme-labelled IgG used in the present study were chosen based on preliminary experiments. Further dilutions of these reagents resulted in weak reactions. The virus was detectable at $10^{-7}$ dilution of banana leaf sap, 5 μl/ml (1 μl/well) of pseudostem sap exudate, 100 ng/ml (20 ng/well) of purified CMV-banana and 500 ng/ml (100 ng/ml) of purified CMV-banana diluted with healthy banana leaf sap in all three tests (Table 1). However, the absorbance values were marginally higher in DAC-ELISA when compared to other two tests. Dilutions of the purified virus with healthy banana leaf extract resulted in decreased sensitivity of virus detection by about five times. Among three forms of ELISA, the sensitivity of DAC-ELISA was compared with local lesion bioassay and dsRNA analysis tests for detection of CMV in banana (Table 2). The banana leaf samples and pseudostem sap exudates that were positive by DAC-ELISA were also positive by the other two tests. In the bio-assay test, cowpea plants inoculated with both infected banana leaf extract and pseudostem sap exudate produced necrotic local lesions after three days of sap inoculation [23 to 34 lesions/primary leaf]. The dsRNA extracted from both infected leaf and pseudostem sap exudate was resolved into 3 bands (Figure 1) with Mr of RNA 1–2.56, RNA 2–2.24 and RNA 3–1.66 × $10^6$ d. No such bands were observed in the corresponding healthy plant samples.

Biological, immunological and molecular techniques are used for diagnosis of CMV-caused diseases[11]. The choice of these techniques is based on expertise and laboratory facilities available, specificity, sensitivity, cost and time factor. Among serological techniques agargel diffusion and ELISA based ones were used for detection of CMV in various plant samples.

In the present investigation, we evaluated both direct (DAS-ELISA and SRDAC-ELISA) and indirect (DAC-ELISA) forms of ELISA for detection of CMV in banana. Sampling from banana by way of collection of pseudostem sap exudate by pinpricking appears simple and ideal in large-scale testing of banana plants/planting suckers (sometimes without leaves) when compared to collection of leaf samples and preparation of extracts from them.

In the present studies the virus was detectable at $10^{-7}$ dilution of banana leaf sap, 5 μl/ml of pseudostem sap exudate and 100 ng/ml of purified CMV-banana in all three ELISA tests. During the extraction of banana

leaves for CMV indexing, samples that oxidize despite the presence of reducing agents can produce false positive reactions in ELISA[11]. These reactions can be minimized by thorough washing of the mucilagenous antigen samples from the wells of the plate before adding the next reagent.

Even though there is no significant difference in the sensitivity levels of virus detection with three tests, the reagents and time required to carry are varied. DAC-ELISA is economic, requires about 5–6 h and is more versatile as the test can be automated and commercialized for application to crops like banana.

In laboratories with minimal facilities, more glasshouse and time (3–4 days) are not the criteria, detection of CMV in banana by bioassay on local lesion hosts like cowpea or green gram (*Phaseolus aureus*) appears suitable routine practice. Further, the other four viruses known to infect banana did not infect these local lesion hosts and thus bioassay is useful for distinguishing CMV from other viruses of banana.

The dsRNA analysis has been used as one of the criteria for identifying the various isolates of CMV in Australia[12]. The application of this technique requires more time (2–3 days), expertise and expensive laboratory facilities. Moreover it is suitable for detecting the virus in a small number of samples. The samples that were positive by DAC-ELISA are also positive by the other two tests (Table 2). Finally we conclude that DAC-ELISA is a suitable test for routine application in large-scale testing of banana encountered in plant quarantine, in planting material (suckers) certification programmes and in banana field surveys.

1. Jeger, M. J., Eden-Green, S., Thresh, J. M., Jhanson, A., Waller, J. M. and Brown, A. E., in *Bananas and Plantains* (ed. Gowen, S.), Chapman and Hall, London, 1995, pp. 316–381.
2. Thomas, J. E. and Dietzgen, R. G., *J. Gen. Virol.*, 1991, **72**, 217–224.
3. Bateson, M. F. and Dale, J. L., *Arch. Virol.*, 1995, **140**, 515–527.
4. Singh, S. J., *Curr. Sci.*, 1988, **57**, 1248–1249.
5. Avgelis, A., *J. Phytopathol.*, 1987, **120**, 20–24.
6. Dale, J. M., *Adv. Virus Res.*, 1987, **33**, 301–324.
7. Bouhida, M. and Lockhart, B. E., *Phytopathology*, 1990, **80**, 981.
8. Mali, V. R. and Rajegore, S. B., *Phytopathol. Z.*, 1980, **98**, 127–136.
9. Mohan, S. and Lakshmanan, P., *Phytoparasitica*, 1988, **16**, 281–282.
10. Patel, K. V. and Mali, V. R., *Indian Phytopathol.*, 1983, **36**, 443–447.
11. Palukaitis, P., Roossinck, M. J., Dietzgen, R. G. and Francki, R. I. B., *Adv. Virus Res.*, 1992, **41**, 281–348.
12. Pares, R. D., Gillings, M. R. and Gunn, L. V., *Intervirology*, 1992, **34**, 23–29.
13. Piazolla, P., Diaz-Ruiz, J. R. and Kaper, J. M., *J. Gen. Virol.*, 1979, **45**, 361–369.
14. Singh, Z., Jones, R. A. C. and Jones, M. G. K., *Plant Dis.*, 1995, **73**, 713–716.
15. Srivastava, A., Raj, S. K., Haq, Q. M. R., Srivastava, K. M., Singh, B. P. and Sane, P. V., *Indian J. Exp. Biol.*, 1995, **33**, 986–988.
16. Hansen, M. A. and Wick, R. L., *Adv. Plant Pathol.*, 1993, **10**, 65–126.
17. Estellita, S., Radhakrishnan, T. C. and Sheela Paul, T. Abstract in X Annual Convention of Indian Virological Society, Trivandrum, 1995, p. 12.
18. Kiranmai, G., M Sc Dissertation, S. V. University, Tirupati, 1989.
19. Walkey, D. G. A., *Applied Plant Virology*, John Wiley and Sons, New York, 1985, p. 113.
20. Brunt, A., Crabtree, K. and Gibbs, A., in *Viruses of Tropical Plants*, CAB International, Oxon, 1990, pp. 225–229.
21. Rajeshwari, R., Iizuka, N., Nolt, B. L. and Reddy, D. V. R., *Plant Pathol.*, 1983, **32**, 197–205.
22. Clark, M. P. and Adams, A. N., *J. Gen. Virol.*, 1977, **34**, 83–106.
23. Clark, M. F. and Barjoseph, M., *Methods Virol.*, 1984, **7**, 51–85.
24. Sreenivasulu, P., Pinnow, D. L. and Demski, J. W., *The University of Georgia Research Report*, 1995, **628**, 1–9.
25. Hobbs, H. A., Reddy, D. V. R., Rajeshwari, R. and Reddy, A. S., *Plant Dis.*, 1987, **71**, 747–749.
26. Diaz-Ruiz, J. R. and Kaper, J. M., *Prep. Biochem.*, 1978, **8**, 1–17.
27. Sambrook, J., Fritsch, E. F. and Maniatis, T., *Molecular Cloning – A Laboratory Manual*, Cold Spring Harbor, New York, 1989, vol. 1, p. 6.3.

ACKNOWLEDGEMENT. The senior author thanks USDA PL480 project authorities for fellowship to carry out this work under Project no. In-ARS-718.

Received 6 May 1996; revised accepted 17 October 1996

# Thin layer problem in geoelectrics

**I. B. Ramaprasada Rao, Ram Raj Mathur and S. Srinivas**

Centre of Exploration Geophysics, Osmania University, Hyderabad 500 007, India

**Inferences in the influence of a thin conductive layer on the controlled source audiofrequency magnetotelluric (CSAMT) Cagniard response as compared to magnetotelluric (MT) soundings are studied. For this purpose, $H_r$ and $E_\phi$ components are computed due to a horizontal electric dipole (HED) over a layered media without imposing farfield or nearfield conditions for the CSAMT method. The computation is carried out in a cylindrical coordinate system using Hankel transform of Bessel functions of order zero and unity with a 100 point digital filter.**

**The root mean square (RMS) deviations between the CSAMT response with and without thin conductive layer, in the frequency ranges of 0.125 Hz to 4096 Hz are computed. A similar scheme of computation is implemented for the MT response. This difference is uniformly higher for the CSAMT within the range of parameters investigated and hence the CSAMT method is preferable over the MT method for delineation of the thin intermediate conductive layer. Conversely, if a lesser influence of the thin conductive layer is desired, MT soundings are preferred over CSAMT studies.**