# MANAGEMENT OF COWPEA APHID-BORNE MOSAIC VIRUS

Thesis submitted in part fulfilment of the
requirements for the degree of Doctor of
Philosophy in Plant Pathology to the
Tamil Nadu Agricultural University,
Coimbatore.

By

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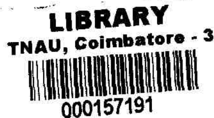
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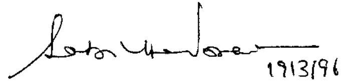

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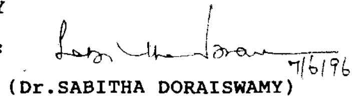

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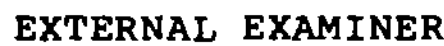

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Date: 7/6/96

***Dedicated to my beloved Parents***

# *ACKNOWLEDGEMENT*

# ACKNOWLEDGEMENT

The author wishes to place on record his profound sense of gratitude and sincere thanks to Dr.Sabitha Doraiswamy, Professor, Department of Plant Pathology and Chairperson of the Advisory Committee for her invaluable and adroit guidance, kind help, constant encouragement and sustained interest evinced throughout the course of this investigation.

The author is grateful to Dr.V.Mariappan, Retired Professor and Head, Department of Plant Pathology who earlier as Chairman of the Advisory Committee had helped a lot during the initial stages of this study.

The author is gratefully indebted to the members of the Advisory Committee Dr.B.Thayumanavan, Professor and Head, Department of Biochemistry, Dr.R.Rathnaswamy, Professor and Head, Department of Pulses, School of Genetics and Dr.P.Balasubramanian, Associate Professor, Centre for Plant Molecular Biology, for their help and valuable suggestions during various phases of this work.

The author wants to offer his deep sense of gratitude to Dr.G.Santharam, Professor, Department of Agricultural Entomology, for his sincere help, valuable guidance and training given during the electron microscopic studies.

The author wishes to record his heartfelt thanks and indebtedness to Mrs. Jayanthi Chinnusamy and Dr.C.Chinnusamy for their help to conduct the field trials.

The author is gratefully indebted to Dr.K.C.George, Professor and Head, Department of Statistics, College of Veterinary and Animal Science, for his valuable suggestions given during the statistical analysis of the data and Smt.K.D.Santha Bai and Mr.K.Prasadan for their help during analysis.

The help rendered by Mr.Khem Bhadur Pun, Viswanathan, Scientist, Sugarcane Breeding Institute, Mr.Johnkutty, Mr.T.Premanathan, Mr.Biju Joseph and Dr.C.Gokulapalan, Mr.S.Elangovan and B.Balasubramanian is memorable and it is gratefully acknowledged. Student friends, Reji Jacob Thomas, M.Joy, Sathyanarayanan, Harindran, Ajoy Sukumaran, S.Sriram, and A.Ramanathan are immeasureably indebted for their sincere help during the course of this investigation.

The author is extremely thankful to ICAR for awarding Senior Research Fellowship during the course of the study and Kerala Agricultural University for sanctioning the study leave.

The assistance and cooperation offered by all the members of the Department of Plant Pathology are gratefully acknowledged.

Words seem to be inadequate to record his heartfelt gratefulness to his beloved wife Mrs.M.R.Rajasree and his son U.Anand, for their tireless support, affectionate encouragement and unbounded inspiration which culminated into the preparation of this thesis.

The author remains thankful to Tamil Nadu Agricultural University for providing facilities to undertake this valuable investigation.

# *ABSTRACT*

ABSTRACT

MANAGEMENT OF COWPEA APHID-BORNE MOSAIC VIRUS

by

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Cowpea aphid-borne mosaic virus disease commonly occurring in Tamil Nadu was investigated. The symptoms appeared as typical mosaic mottling with dark green and light green patches sometimes showing vein banding symptoms. The virus was transmitted through seeds, mechanical means and by *A.craccivora*, *A.gossypii* and *M.persicae*. The virus-vector relationship showed that a short acquisition access period of 5 minutes made the aphids viruliferous. An inoculation access of one minute was only required for the transmission by the vector. Preacquisition starvation of one hour showed a maximum infection of 88 per cent

and preinoculation starvation decreased the per cent infection. A single aphid was able to transmit the disease and the viruliferous nature of the vector was lost after 2 h.

The host range of the virus was confined to four families *viz*., Amaranthaceae, Chenopodiaceae, Labiatae and Leguminosae. Anatomical studies revealed the severe deformation of the infected tissues.

The virus was detected by DAC-ELISA and the virus isolate showed strong reaction to CAMV polyclonal and monoclonal antiserum. There was only a weak reaction with BICMV monoclonals. Detection using NCM-ELISA also showed that the virus could be detected even at 1:1000 dilution in leaf extract and in body fluid of single viruliferous aphid. Electron microscopic detection revealed the presence of flexuous filamentous particles of $\sim$750 nm. Immunosorbent electron microscopy also showed heavy trapping of virus when polyclonal antisera and potyvirus group antisera were used.

The physiology of virus infected plants showed that chlorophyll content was reduced in infected plants. The level of carbohydrate was reduced in infected plants. The total soluble protein content showed a significant increase in infected susceptible varieties. The protein profile of seed proteins also

showed marked difference in healthy and diseased plants. The electrophoretic analysis of PR-proteins revealed the presence of three noval proteins with molecular weights of 34, 31 and 23 kDa in treated plants. Isozyme studies on native gel showed distinct difference between resistant and susceptible genotypes in their isoPOs activity. There was an increase in RNA content and decrease in the level of DNA in infected susceptible varieties. The assay of enzymes revealed that all the enzymes PO, PPO, PAL and chitinase showed an increase in activity in plants treated with AVP and in plants inoculated after treatment with AVP. Inoculation with CAMV alone showed an increase in the level of all enzymes analysed. There was an early induction of total phenol and OD phenol in inoculated plants.

Under glasshouse conditions antiphytoviral chemicals *viz*., barium chloride, ammonium molybdate, thiouracil and copper sulphate were found to inhibit the CAMV infection. Among the plant species screened for antiviral properties, the leaf extracts of *C.annum*, *B.rubra*, *C.bonplandianum*, *I.carnea* and *M.jalapa* were found to be superior. Mixing plant extracts with virus inoculum also showed the inhibition of the disease. The active principle showing antiviral property was found to be protein fraction. The protein fractions from MJLE, BSLE, PCLE and ICLE were found to be highly effective against CAMV. The application of partially purified *Bougainvillea* antiviral protein

(BAP) on cowpea seedling accelerated the activities of PO, PPO and PAL enzymes.

Management of CAMV disease underfield condition showed that four leaf extracts BSLE, MJLE, PCLE and ICLE, two non-edible oils (neem and mahua) and an insecticide (monocrotophos) reduced the disease incidence and aphid population. The treatments improved the growth of the plants and increased the yield.

Out of 332 germplasm tested, 40 showed resistant reaction. Seventy eight were tolerant, 90 susceptible and the remaining 124 were highly susceptible.

In vitro callus culture and regeneration of cowpea revealed that maximum callusing was obtained in MS medium supplemented with 2,4-D 2 mg $l^{-1}$ + Kn 1.0 mg $l^{-1}$ when leaf was used as explant and hypocotyl explant gave maximum callusing in MS + 2,4-D 2.5 mg $l^{-1}$ + Kn 1.0 mg $l^{-1}$. There was no regeneration from any of these callus when transferred to regeneration medium.

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# *INTRODUCTION*

# CHAPTER I

# INTRODUCTION

Cowpea, Vigna unguiculata (L.) Walp., is an important high-protein food crop in many tropical and subtropical countries. It is indigenous to Africa, from where it was introduced into other tropical and subtropical countries. It is now grown in India, South East Asia, the West Indies, the USA, Australia and parts of Europe and South America.

In Tamil Nadu it is cultivated in 1.56 lakh hectares with an annual production of 0.32 lakh metric tonnes and an average productivity of 205 kg/ha (AGROSTAT, 1994). In the tropical subsistence agriculture, grain legume yields are often very low. Many factors contribute to these very low yields; in some countries diseases are often the cause of reduced production (Singh and Allen, 1980; Allen, 1983; Thottappilly, 1992). Among pathogens, several viruses have been associated with the poor yields. Virus diseases are a major threat to the large-scale production of this crop in several countries. Geographical distribution of several cowpea viruses is not yet accurately determined, and many virus diseases remain to be identified.

In India, cowpea is known to be affected by several virus diseases, the most important of which is caused by Cowpea Aphid-borne Mosaic Virus (CAMV). CAMV was first described in Italy (Lovisolo and Conti, 1966). CAMV is of economic importance since it causes an yield loss of 15-87 per cent under natural field conditions and from 29 to 40 per cent under artificial inoculations in the field (Mali and Thottappilly, 1986; Thottappilly, 1992). Because of the importance of CAMV in the cultivation of cowpea in Tamil Nadu and Kerala, this study was initiated to obtain additional information with the following objectives.

1. To study the transmission by mechanical, insect vector and seed
2. To determine the virus-vector relationship
3. To assess the varietal resistance of cowpea to CAMV
4. To analyse the isozymes to correlate with disease resistance
5. To characterize the antiviral nature of plant derivatives
6. To study the effect of plant derivatives on the survival of insect vectors
7. Management of CAMV disease using chemicals, antiviral suppressors and plant derivatives under field condition

# *REVIEW OF LITERATURE*

# CHAPTER 2

# REVIEW OF LITERATURE

## 2.1 DISTRIBUTION

Lovisolo and Conti (1966) described for the first time cowpea aphid-borne mosaic virus (CAMV) from Italy. An African isolate, described from Kenya and inducing an irregular, angular broken mosaic, has been referred to as the 'African strain' (Bock, 1973). Another isolate from East Africa described as the 'African vein-banding strain' (Bock, 1973) induces a broad dark-green vein-banding. A third African isolate, distinguished by Bock (1973) and referred to as 'African mild strain', induces a very mild mottle with little or no effect on plant growth. In addition it occurs in Egypt, Kenya, Morocco, Nigeria, Tanzania, Uganda, Zambia (Phatak, 1974; Ladipo, 1976; Fischer and Lockhart, 1976; Rossel, 1977; Patel and Kuwaite, 1982), Asia (India, Iran, Japan and the Philippines) (Tsuchizaki *et al*., 1970; Phatak, 1974; Kaiser and Mossahebi, 1975; Beningo and Hedayat, 1977; Mali *et al*., 1983), Australia (North Queensland) (Benhneken and Maleevsky, 1977) and Europe (Italy) (Lovisolo and Conti, 1966).

Blackeye cowpea mosaic virus (BlCMV), a virus very similar or identical to CAMV, was originally reported from Florida, USA (Anderson, 1955; Zettler and Evans, 1972; Lima *et*

al., 1979; Purcifull and Gonsalves, 1985). CAMV and BlCMV, although serologically related, are not identical (Taiwo and Gonsalves, 1982; Taiwo et al., 1982) and so cowpea varietal reactions to them differ. However, recent studies indicate that CAMV and BlCMV are two different potyviruses (Huguenot et al., 1993; 1994).

## 2.2 SYMPTOMATOLOGY

Symptoms of the isolate first described from Italy (Lovisolo and Conti, 1966) - the so called 'European strain' consist of a severe distorting mosaic in cowpea. Bock (1973) described the symptoms produced by three isolates from Africa. An isolate first identified from Kenya induces an irregular, angular broken mosaic. Another isolate from East Africa induces a broad dark green vein-banding. A third African isolate induces a very mild mottle with a little or no effect on plant growth. Bock and Conti (1974) noticed variable amounts of dark green vein banding or interveinal chlorosis, leaf distortion, blistering and stunting of cowpea plants infected with CAMV. Raheja and Leleji (1974) reported the symptoms which include widespread mottling (giving the field a yellowish appearance), chlorosis between veins and vein-banding. In some cases chlorotic mottling resulted in distinct patterns of light and dark green areas. Later, edges of infected leaves curling downwards, puckering or

developing large rust coloured necrotic areas. Plants become stunted, bushy and the flowering was retarded. Fischer and Lockhart (1976) found that the infected plants commonly showed mosaic patterns, leaf bumping and distortion and serious stunting. Beningo and Hedayat (1977) described include dark green vein-banding, mottling, puckering and distortion of leaves and stunting of plants. Ramiah (1978) reported that CAMV caused mosaic mottling, puckering, cupping and malformation of leaves. Mali and Kulthe (1980a) found that symptoms on primay leaves were either chlorotic bands along the major veins or line pattern or no symptoms. These were followed in secondary leaves with various types of mosaic symptoms including puckering and distortion of leaf lamina. The infected adult plants exhibited severe puckering, mottling and defoliation of the trifoliates, and extensive necrotic streaks in the stem, sometimes leading to drying of the entire plant (Patel and Kuwaite, 1982). Symptoms caused by CAMV include severe mosaic, with the severity dependent on host cultivar and virus strain (Rossel and Thottappilly, 1985; Thottappilly and Rossel, 1985; Thottappilly and Rossel, 1992). Patil and Gupta (1992a) stated that the seed-borne mosaic virus of cowpea caused mosaic, vein clearing, vein-banding, puckering, distortion and reduction in the size of the leaves of cowpea. Thenmozhi (1994) described the symptom due to CAMV as interveinal chlorosis and varying degrees of vein-banding of leaves. The leaves of severely diseased plants were blistered,

distorted and puckered. Plants infected in the early stages were severely stunted and produced a few or no pods. Pods produced by severely diseased plants were a few and small with shrunken seeds.

## 2.3 THE VIRUS

CAMV was characterized by flexuous, filamentous particles which were ≈ 750 nm long (Lovisolo and Conti, 1966; Bock, 1973; Bock and Conti, 1974; Kaiser and Mossahebi, 1975; Lima et al., 1979). Fischer and Lockhart (1976) reported that partially purified preparations from deep frozen leaf material of systemically infected cowpea plants contained filamentous rods with a calculated mean length of about 765 nm. Behncken and Maleevsky (1977) found that CAMV particles were flexuous and filamentous, ranging from 727 ± 42 nm. Mali and Kulthe (1980b) revealed that the electron micrographs of negatively stained leaf dip preparations of CM-11 cowpea and Chenopodium amaranticolor infected with the virus were flexuous rods of about 750 nm in length. They also found the similar rod shaped virus particles in cowpea leaves infected with the virus from seed, but not in preparations from healthy leaves of cowpea.

Patel and Kuwaite (1982) also reported that the particles were filamentous in samples identified as CAMV. Same type of results were obtained in asparagus bean (Vigna

*sesquipedalis*) by Chang and Kuo (1983). They observed that CAMV particles were flexuous, rod-shaped measuring a length of 750 nm. Sekar and Sulochana (1983) also found that a virus from mosaic infected cowpea were fluxuous particles of 750 nm in length. Thottappilly and Rossel (1992) also reported the same from Nigeria.

## 2.4 TRANSMISSION OF THE DISEASE

### 2.4.1 *Sap transmission*

Nariani and Kandaswamy (1961) reported that the disease was successfully transmitted to healthy cowpea plants by mechanical inoculation with the expressed juice from the mosaic affected plants by leaf rubbing method using carborundum powder as an abrasive. They found that the symptoms appeared on the inoculated plants in about 7-10 days after inoculation in the form of vein clearing followed by mosaic mottling of the leaves. Mechanical transmission of CAMV was reported by Chenulu *et al*. (1968). Govindaswamy *et al*. (1970) found that the virus was easily transmitted by sap and the inoculated plants exhibited disease symptoms within 7 to 14 days of inoculation. Bock (1973) could successfully transmit CAMV to cowpea plants by mechanical inoculation. Khatri and Singh (1974) could readily transmit the virus through sap inoculation to healthy cowpea plants and local lesion hosts. Iwaki *et al*. (1975) found that the spread of CAMV

was possible by sap inoculation. Sharma and Varma (1975b) found that three sap transmissible viruses from cowpea commonly occuring in Western India could be mechanically transmitted to healthy plants of same species using 0.05 M phosphate buffer (pH 7.0).

Fischer and Lockhart (1976) found that CAMV was transmissible by sap inoculation using 0.05 M phosphate buffer, pH 7.1. Cowpea varieties CM-11, K-11 and JL were successfully inoculated with a seed-borne potyvirus causing mosaic of cowpea by mechanical inoculation. The test plants were inoculated by conventional leaf rub method with crude sap extracted from virus infected leaves in cold 0.01 M Tris buffer, pH 7.0 (Mali and Kulthe, 1980a; 1980b). Patel and Kuwaite (1982) described that CAMV could be transmitted to healthy test plants by sap transmission. Further studies have also confirmed the mechanical transmission of CAMV (Atiri et al., 1984; Guo et al., 1984; Mazyad et al., 1984; Mali et al., 1988; Atiri and Thottappilly, 1984; Revathi, 1991; Patil and Gupta, 1992a; Thottappilly and Rossel, 1992; Nain et al., 1994).

2.4.2 Seed transmission

Many plant viruses were found to be transmitted through seeds of the infected plants. Some of the viruses infecting cowpea were also reported to be transmitted through seeds.

Ladipo (1977) carried out investigations on CAMV transmission in seeds from infected cowpea plants and found that it was dependent on the cultivar or line. Among the 19 varieties tested, he reported the highest seed transmission of 20.9 per cent in West Bred cowpeas. Similar report was made by Aboul-Ata et al. (1982).

Gupta and Summanwar (1980) studied the location of two mosaic viruses in cowpea seeds and reported that the virus was located mostly in the plumule bud and cotyledons of diseased seeds. Mali and Kulthe (1980b) also found that the virus was transmitted through the seeds of CM-11 cowpea variety. They reported that the level of seed transmission in CM-11 cowpea was 41.6 per cent. Mazyad et al. (1984) reported from Egypt that CAMV was seed transmissible.

Sharma and Varma (1986a,b) proved that field grown plants of Pusa Dophasli inoculated 10 days after sowing produced 18-25 per cent seeds with cowpea banding mosaic virus. Sekar and Sulochana (1988) studied the seed transmission of black eye cowpea mosaic virus (BICMV) in two cowpea varieties and reported that the per cent seed transmission of BICMV in Pusa Dophasli cowpea varied from 1.4 - 6.7 and 18.3 per cent in Cp-455. They also found that the distribution of infection among seeds of single pod was uniform.

Silveira *et al*. (1988) carried out investigations on the virus transmission through commercial cowpea seeds and found that the seeds were infected by a potyvirus. They reported that the virus was serologically related to BICMV and the transmission rate ranged from 0.1 to 0.3 per cent. Mali *et al*. (1988) found that the level of seed transmission ranged from 0 - 22.6 per cent for BICMV and 0 - 18.5 per cent for CAMV. Mali *et al*. (1989) confirmed the seed transmission in 28 of 60 cowpea cultivars tested. They reported that the seed-borne viruses identified included BICMV, which was seed transmitted at the rate of 7.8 to 41.8 per cent and CAMV at a range of 3.1 to 20 per cent. Fidan and Yorganci (1990) showed the presence of virus in infected seeds of cowpea serologically. Hampton *et al*. (1992) could detect CAMV in 9 out of 23 infected seed lots by ELISA technique. Gillaspie *et al*. (1993) found that BICMV was seed-borne in 4 of 7 cowpea seed lots tested and reported that there was an incidence of 0.4 to 50 per cent. Nain *et al*. (1994) while investigating on the growth stages of cowpea and seed transmission of the viruses found that there was an inverse relationship between per cent seed transmission and age of the plant at inoculation. They also reported that one week old plants were more prone to seed transmission.

### 2.4.3 Insect transmission

CAMV was reported to be transmitted by a number of vectors. Govindaswamy *et al*.(1970) reported that a mosaic disease of cowpea in Coimbatore was found to be transmissible by *Myzus persicae*, *Aphis craccivora*, *A. gossypii* and *A.evonymii*. Khatri and Singh (1974) reported three vectors transmitting a mosaic disease of cowpea, namely *A. craccivora*, *A. gossypii* and *M. persicae*. Iwaki *et al*. (1975) found that CAMV was easily transmitted by *A. craccivora*. Kaiser and Mossahebi (1975) while investigating the transmission of CAMV, found that *A. craccivora*, *Acryrthosiphon pisum* and *Aphis sesbaniae*, could transmit the virus to healthy cowpea plants in a stylet-borne manner.

Fischer and Lockhart (1976) reported that a strain of CAMV isolated from cowpea in Morocco was transmitted in a non-persistent manner from cowpea to cowpea by *M. persicae*. Ramiah (1978) could transmit CAMV to cowpea by *A. craccivora* more efficiently when compared to *M. persicae* and *A. gossypii*. Transmission of CAMV was found to be by *A. gossypii* in CM-11 cultivar of cowpea (Mali and Kulthe, 1980a). They found that the vector transmitted the virus from cowpea to cowpea with brief acquisition and transmission feeding periods indicating a non-persistent manner of transmission. CAMV was readily transmitted in a non-persistent manner by *A. craccivora*, *A.fabae*, *A. gossypii*

and M. persicae (Atiri, 1984; Atiri et al., 1984; 1986). Guo et al. (1984) reported that CAMV from Vigna sesquipedalis could be transmitted by M. persicae, A. gossypii and A. craccivora in a non-persistent manner. Santos et al. (1984) while investigating two potyvirus isolated from cowpea found that it could be transmitted by M. persicae and Aphis citricola. Li et al. (1990) found that the virus could be transmitted by aphids (M. persicae, A. gossypii, A.fabae and A. craccivora) in a non-persistent manner. Thottappilly and Rossel (1992) in their review of virus diseases of cowpea in tropical Africa reported that CAMV was transmitted non-persistently by A. craccivora, A. gossypii, Aphis spiraecola, Aphis medicaginis, Macrosiphum euphorbiae, M. persicae, Rhopalosiphum maidis and Cerataphis palmae. Roberts et al. (1993) while investigating the ability of population of adult apterous A. craccivora, A. gossypii and A.citricola to transmit CAMV to cowpea found that the infection level with A. gossypii was higher than with A. craccivora and both were more efficient than A.citricola although the differences were not significant. Nain et al. (1994) reported that CAMV was non-persistently transmitted by A. gossypii, A. craccivora and M. persicae.

## 2.5 LOSSES DUE TO THE DISEASE

Nariani and Kandaswamy (1961) observed that the pods from plants were usually small and shrunken, containing only a few shrivelled seeds, which in most cases were not viable. They also found that the yield was considerably reduced. An yield loss of 44-80 per cent was observed in glasshouse and field inoculation with CAMV. It was also found that there was considerable reduction in seed yields from infected plants by 13-83 per cent (Kaiser *et al*., 1968). Raheja and Leleji (1974) reported a complete loss of an irrigated cowpea crop in northern Nigeria was tentatively attributed to CAMV infection.

Kaiser and Mossahebi (1975) found that in plants naturally infected with CAMV in field trials, yields were reduced by 13-87 per cent. They also found that the pods and seeds from many lines were deformed, reduced in size, and discoloured. The germination of seeds from infected plants were also decreased. They could find that the yields of cowpea plants inoculated mechanically in the field were reduced by 29 to 44 per cent. They also reported that there was little difference in yields of plants infected in the seedling or flowering stages, within cowpea varieties. Gilmer *et al*. (1975) reported that an early infection (7 days after emergence) reduced yields by 40-60 per cent but late infection (after flowering) caused reduction of

only 5-10 per cent. Sharma and Varma (1976) found that the yield of infected plants was reduced by 41.8 per cent.

Fernandez and Lastres (1983) reported that cowpea mosaic virus caused yield reduction of 64-75 per cent in four varieties in inoculation trials. They found that the yield components most affected were number of pods per plant and seed yield per plant. The virus infection reduced the number of flowers, pods, seed output, size and weight of pods and seeds (Singh and Singh, 1985). They also found that the reduction was greater in early inoculated plants and the loss in yield was mainly attributed to the reduction in the rate of photosynthesis. Frison (1988) recorded an yield loss of upto 100 per cent in cowpea plants infected with CAMV.

Patil and Gupta (1988) found that in glasshouse and in field, the potyvirus significantly reduced the height, fresh and dry weights of shoots and roots on inoculation. The number of pods per plant was significantly affected, but not the number of seeds per pod. Gonsalves and Lima (1988) reported that there was considerable reduction in plant height, leaves per plant, dry weight of stem, root and leaves, pod length, pods per plant, seeds per pod, seeds per plant and 100-seed weight of cowpeas inoculated with the virus.

Rao *et al*. (1989) suggested that yield loss due to virus infection in cowpea may be partially due to the reduced rate of photosynthesis. Patil and Sayyad (1991) found that the infection due to the virus reduced the number of nodules, fresh weight, and dry weight of plants. Atiri and Mih (1992) found that simultaneous inoculation with CMV and BICMV and inoculation at 4 days intervals decreased dry matter and yield more than the plants inoculated at 20 days intervals. Patil and Gupta (1992b) while investigating on the effect of seed-borne mosaic virus on growth and yield of cowpea found that the infection reduced plant height, fresh and dry weight of shoot and root in comparison with healthy plants in the field and glasshouse conditions. They also found that the number of pods per plant were reuced but not the number of seeds per pod. They observed that the yield losses of cv. Pusa-4 were 42.4 per cent under field conditions. Roberts *et al*. (1993) found that CAMV and CMV were the important pathogen of cowpea crops. They also found that these two viruses recorded as the possible cause of yield loss of upto 85 per cent (CAMV) and 14 per cent (CMV) in cowpeas. Thenmozhi (1994) reported that cowpea plants when inoculated with CAMV 10 days after sowing were severely stunted and produced no flowers and pods. The plant height, dry weight of roots and shoots, number of pods, grains and grain weight per plant were directly proportional to the time of inoculation.

## 2.6 VIRUS-VECTOR RELATIONSHIP

The virus-vector relationship of CAMV was worked out by many authors. They observed that CAMV was readily transmitted by aphids in a non-persistent manner (Vidano and Conti, 1965; Bock, 1973; Kaiser and Mossahebi, 1975; Mali and Kulthi, 1980b; Atiri, 1984; Guo *et al*., 1984; Santos *et al*., 1984; Atiri and Thottappilly, 1984; Murphy *et al*., 1987; Li *et al*., 1990; Ndiaye *et al*., 1993; Roberts *et al*., 1993).

Nariani and Kandaswamy (1961) could get successful transmission of the disease by starving the aphids for 2 h prior to an acquisition feeding period of 30 minutes. The insects were allowed to feed for 24 h on the test plants. Govindaswamy *et al*. (1970) reported that a single aphid could transmit the virus to healthy plants. The aphids were found to acquire the virus even within a second and transmit the same. Murugesan and Janaki (1972) while investigating the relationship of cowpea mosaic virus with the vector *M. persicae* found that the virus could be transmitted to healthy cowpea plants even by a single aphid. They found that the maximum infection was obtained with 15 aphids and after 1 h acquisition feeding, although the virus could be acquired in one second. They reported that a preliminary fasting upto 2 h increased the efficiency of transmission only when followed by short acquisition feeding (upto 5 minutes). They

also found that post-acquisition fasting decreased the efficiency of the vector and the virus was retained only upto 4 h.

Sharma and Varma (1977) showed that a single aphid could transmit the virus. They found that an increase in the number per test plant resulted in a corresponding increase in per cent transmission. They also found that the aphids transmitted the virus even without pre-acquisition fasting. They gave an optimum period of 3 h. The transmission was maximum after 5 minutes acquisition feeding and in inoculation access time of 30 minutes. The acquisition, inoculation and transmission thresholds were 20, 25 and 50 seconds respectively. They could find that the infectivity was lost after 2 h post-acquisition fasting. Atiri (1984) reported that CAMV could be readily transmitted in a non-persistent manner by A. craccivora to four cowpea lines. He found differences in optimum pre-acquisition fasting, acquisition feeding, inoculation feeding periods and retention times on the four lines tested. He proved that the differences was due to the nature of aphid probing.

Revathi (1991) obtained maximum transmission of CAMV, with pre-acquisition fasting period of 15 minutes, acquisition feeding period of 20 minutes and inoculation feeding period of 60 minutes with five aphids per plant. Nain et al. (1994) could successfully transmit CAMV using viruliferous aphid nymphs of

A. craccivora, A. gossypii and M. persicae. the nymphs were given 1 h fasting, 5 minutes acquisition access and 24 h infection access period for effective transmission of the virus. Thenmozhi (1994) found that pre-acquisition fasting period of 10 minutes, acquisition feeding period of 20 minutes and inoculation feeding period of 50 minutes with nine aphids per plant to get maximum transmission of CAMV. She also reported that even a single aphid could transmit CAMV to an extent of 5 per cent.

## 2.7 HOST RANGE OF CAMV

Nariani and Kandaswamy (1961) while investigating the mosaic disease of cowpea found that Vigna sesquipedalis, Phaseolus aureus, P.mungo, P.limensis, P.lunatus and Canavalia ensiformis were systemic hosts of the virus. Chenulu et al. (1968) found that the host range was restricted to some Leguminosae though distinct local necrotic lesions were produced on Chenopodium album and C.amaranticolor. Govindaswamy et al. (1970) reported that C.ensiformis, Phaseolus vulgaris, Vicia faba, Cyamopsis tetragonoloba belonging to Leguminosae, C.album and C.amaranticolor belonging to Chenopodiaceae were found to be infected by the virus.

East African isolates of CAMV had a wide host range comprising of 19 leguminous and 11 non-leguminous hosts (Bock,

1973). Zettler and Evans (1972) found that the virus infected *Pisum sativum*, *P.vulgaris*, *Melilotus alba*, crimson clover and broad bean. Bock and Conti (1974) reported that *C.amaranticolor*, *Glycine max*, *Ocimum basilicum*, *P.vulgaris* and *P.sativum* were the diagnostic hosts of CAMV. Khatri and Singh (1974) found that CAMV could produce systemic symptom on *V.unguiculata*, *P.vulgaris* and *Crotalaria juncea* and local necrotic lesions developed on *C.album* and *C.amaranticolor*.

CAMV was also infectious to other leguminous hosts *viz*., pigeon pea, sunnhemp, French bean, peas, fenugreek, mung-bean, guar, urdbean, chickpea, limabean, lentil, asparagus-bean, soybean, *Calapagonium mucanoides*, *Cassia occidentalis*, *Crotolaria brevidens*, *C.spectabilis*, *C.usaramoensis*, *C.paulina*, *Desmodium tortuosum*, *Dolichos uniflorus*, *Lens esculenta*, *Macroptilium lathyroioides*, *Medicago scutellata*, *Mucuna pruriens*, *Sesbania speciosa*, *Trifolium incarnatum*, *T.subterraneum*, *Vigna oblongifolia* var. *oblongifolia*, *V.luteola*, *V.membranaceae*, *V.schimperi*, *V.triloba* and *V.umbellata* (Phatak, 1974; Kaiser and Mossahabi, 1975; Behncken and Maleevsky, 1977).

Ramiah (1978) reported 11 and 6 out of 57 plant species tested, produced local lesions and systemic symptoms with CAMV respectively. Mali and Kulthi (1980a) found that CAMV had a restricted host range and confined to 10 out of 42 species tested

in Amaranthaceae, Chenopodiaceae and Leguminosae. Among the host species tested, Patel and Kuwaite (1982) found that C.amaranticolor and C.quinoa produced chlorotic local lesions without systemic symptoms and in Gomphrena globosa the lesions were necrotic. P.vulgaris cultivars 'Pinto' and 'Monroe' gave necrotic local lesion, 'Canadian Wonder' produced chlorotic local lesion and 'Processor' showed mixture of both. In Vigna spp. inoculated, V.aconitifolia L. and V.radiata exhibited systemic mosaic, puckering and systemic necrosis killing the growing shoot and/or the entire plant. Vigna mungo showed mosaic and puckering symptoms. G.max gave mosaic, puckering, vein clearing and stunting. Cajanus cajan, Cicer arietinum, Lablab niger, Voandzeia subteranea and Lens esculentum proved to be symptomless carriers.

Boswell and Gibbs (1983) described the host range of CAMV in G.globosa, C.amaranticolor, C.quinoa, Cucumis sativus, O.basilicum, G.max, P.vulgaris, P.sativum, Trigonella foenum-graecum, Petunia hybrida, N.clevelandii, Tetragonia expansa, Trifolium subterraneum, Vicia faba, P.lunatus and Medicago sativa. Rossel et al. (1985) could isolate CAMV from all ecological zones of Nigeria from cowpeas and also found its presence in Vigna subterranea, Sphenostylis stenocarpa and wild cowpea. Patel and Gupta (1992a) found that the host range was restricted to some species of Chenopodiaceae and Leguminosae.

Gahukar and Kalore (1987) reported that CAMV was confined to a very narrow host range infecting only P.vulgaris and C.amaranticolor. Murphy et al. (1987) found that BICMV infected O.basilicum, P.lunatus, P.vulgaris and V.faba. Nagaraju and Keshavamurthy (1994) reported that the host range of the virus was restricted to 9 species out of 41 tested. The virus produced chlorotic spot and thickening along veins in Cucurbita moschata and Luffa acutangula, systemic mosaic in Cajanus cajan, C.tetragonaloba, Phaseolus aureus and P.vulgaris, chlorotic local lesions on C.amaranticolor and C.quinoa and systemic necrosis on Dolichos biflorus. Eight out of 21 crop plants tested were susceptible to CAMV (Thenmozhi, 1994). She found that Amaranthus polygamous, A.viridis, Desmodium tortuosum, Lablab niger, P.vulgaris, P.sativum, V.mungo and V.radiata as the hosts of CAMV.

2.8 PHYSIOLOGY OF VIRUS INFECTED PLANTS

2.8.1 Dry matter production

Mali et al. (1980) found that there was considerable reduction in shoot and root length of cowpea plants infected with the virus and they could prove that it lowered the total dry weight of shoot, root and nodules, when compared to healthy plants. Fegla et al. (1981) showed that in cowpea, infected with CAMV and CMV, the fresh weight and dry weights of shoot and root

were affected. They found that the reduction was maximum on combined infection. Ohair and Miller (1982) reported that under glasshouse condition and in field experiments there was drastic reduction in total nodule weight, nodule number and plant weight in susceptible cultivars. Patil and Gupta (1988) could find in greenhouse and field, bean common potyvirus significantly reduced the fresh and dry weights of shoot and roots on artificial inoculation of cowpea plants. Gonsalves and Lima (1988) also found the same. Singh _et al_. (1989) found that the virus reduced both net and gross production of dry matter in cowpea plants infected by southern bean mosaic virus (SBMV). Patil and Sayyad (1991) while investigating the cowpea mosaic virus-rhizobium interaction found that it reduced the number of nodules, fresh and dry weight of plants and nodules. Atiri and Mih (1992) reported that CAMV in combination with CMV reduced the dry matter production when inoculated at 4-day interval.

### 2.8.2 Photosynthesis and respiration

There was significant reduction in chlorophyll content in virus infected susceptible variety Co.2 (Ramiah, 1978). Singh and Singh (1985) found that the loss in yield was mainly attributed to the reduction in rate of photosynthesis. Tripathi _et al_. (1987) found that the contents of total chlorophyll, chlorophyll _a_, chlorophyll _b_ and carotenoids were reduced in

infected leaves than in healthy ones. Mayoral *et al*. (1989) found that cowpea mosaic virus reduced the chlorophyll content of infected plants. There was pronounced reduction in chlorophyll content in plants infected with cowpea mosaic virus but the content of carotenoid was unchanged. The total chlorophyll, chlorophyll *a* and *b* increased upto 60 days after inoculation and then declined (Rao *et al*., 1989). They suggested that the yield loss due to virus infection was principally due to a reduced rate of photosynthesis.

Singh *et al*. (1989) found that infection of cowpea by southern bean mosaic increased the rate of respiration. They also reported that the respiration loss of assimilates increased with plant age continuously in infected tissues.

2.8.3 Carbohydrate metabolism

Khatri and Chenulu (1969) found that the reducing sugar content was not appreciably affected by cowpea mosaic virus in resistant and susceptible cowpea cultivars. Padma *et al*. (1976) reported that cowpea mosaic virus infected seeds contained a higher percentage of sugars. Ramiah (1978) reported that there was decreased synthesis of total carbohydrates of infected leaves of susceptible parents. He also observed that the trifoliate leaves showed reduction in the level of carbohydrates commencing

from 10th day after inoculation. Singh and Singh (1984) found that the virus infection decreased total sugar and starch in cowpea cultivars infected with SBMV. Singh and Singh (1987) could find that both southern bean mosaic virus and cowpea mosaic virus reduced the carbohydrate fraction (total, reducing and non-reducing sugars and starch) in cowpea cv. Pusa Dofasli. Sastry and Nayudu (1988) found a higher quantities of carbohydrate in hypersensitive cowpea cultivars infected with tobacco ringspot nepovirus and suggested that the infected area acts as a metabolic sink. Mayoral et al. (1989) reported that the carbohydrate level was much reduced in infected leaf tissues and was conspicuous from healthy leaves.

### 2.8.4 Nitrogen metabolism

Virus infection increased total nitrogen but the difference was less marked in resistant cultivar when compared to susceptible cultivar. There was no qualitative difference in free amino acid composition of leaves of healthy and inoculated plants of either cultivar at any stage of disease development (Khatri and Chenulu, 1974). They also found that some free amino acids accumulated in the resistant cultivar and decreased in the susceptible one. They could find the nitrate reductase activity to a greater extent in the susceptible cultivars following inoculation. Padma et al. (1976) reported that cowpea mosaic

virus infected seeds contained a higher percentage of proteins, than healthy seeds. Ramiah (1978) also found that total nitrogen content was higher in the inoculated plants than that of comparable healthy plants. Singh _et al_. (1978) reported that the virus infection resulted in higher total nitrogen, total protein, nitrate and nitrite nitrogen than in healthy leaves of cowpea infected with SBMV. Mali _et al_. (1980) could find an increase in the total nitrogen content of infected shoots and roots of cowpea infected with TMV-cowpea strain.

Singh and Singh (1981) while investigating the changes in nitrogenous constituents of cowpea fruits due to cowpea mosaic virus found that there was an increase in total nitrogen, protein, and nitrate nitrogen. They observed a decrease in nitrite nitrogen and ammoniacal nitrogen levels in infected Pusa Phalguni fruits.

Ohair and Miller (1982) observed that the cowpea strain of TMV was associated with reduction in nitrogenase activity. They also found that SBMV infection reduced the activity of the same enzyme in susceptible cowpea cultivars. Further studies have also confirmed that virus infection increased the protein yield in cowpea (Singh and Singh, 1984; Singh and Singh, 1987; Yadav, 1988; Yadav and Sharma, 1988; Mayoral _et al_., 1989; Patil and Sayyad, 1991).

2.8.5 Pathogenesis-related proteins (PR proteins)

It has been well known that the hypersensitive response of virus infection is accompanied by the appearance of one or more new infection specific soluble proteins. Such proteins are also accumulated occasionally in plants in response to infection with viroids, fungi and bacteria, and to chemicals. In infected plants these *de novo* synthesized proteins have been named as pathogenesis-related (PR) proteins.

Rottier *et al*. (1980a,b) while investigating protein synthesis in cowpea mosaic virus (CPMV) infected cowpea protoplast, detected eleven polypeptides with molecular weight of 170, 130, 112, 110, 87, 84, 68, 37, 30, 24 and 23 (all x $10^3$) whose synthesis was either induced or stimulated by CPMV infection. They found that 170 x $10^3$ and 30 x $10^3$ species were the first virus-related polypeptides detectable between 9 and 15 h after inoculation. From about 16 h after inoculation, all other virus-related proteins appeared to be synthesized at increasing rates. Coutts and Wagih (1983) observed that inoculation of cowpea leaves and cucumber cotyledons with tobacco necrosis virus (TNV) resulted in a reproducible alterations to the soluble protein profile of both species. They designated the noval host proteins as and fractions in cowpea leaves, induced during virus-elicited necrosis. Yoshikawa *et al*. (1993) reported four PR

proteins in cowpea leaves infected with cucumber mosaic virus and designated CpPR-1, -2, -3 and -4 in the order of faster migration. They also detected CpPRs production at the time of necrotic local lesion appearance and increased as the lesion matured.

2.8.6 Nucleic acid metabolism

The total RNA level was found to be 5.6 and 1.6 times more than that of healthy cowpea and soybean respectively, when they were infected by cowpea mosaic virus and tobacco ringspot virus (Lee *et al.*, 1975). Ramiah (1978) found that in CAMV infected cowpea cultivars, Co.1 and Co.2, the RNA content of trifoliate leaves maintained at higher level at 20 and 40 days after inoculation, when compared to healthy plants. He also found that the levels of RNA in resistant cultivar was not altered at any time after inoculation. In his investigations, the DNA level was also more in infected plants when compared to healthy susceptible plants. In resistant cultivar the variation in DNA content was not significant. Sastry and Nayudu (1988) recorded higher quantities of RNA in hypersensitive cowpea primary leaves infected with tobacco ringspot nepovirus. Infection of *Dolichos lablab* by yellow vein mosaic virus under natural conditions caused a decrease in total DNA content

(Hossain and Haider, 1992). They also found that the RNA content was increased in infected plants when compared to healthy plants.

2.8.7 Enzymatic activities

In hypersensitive reacting plants virus localisation and necrosis is accompanied by alterations to the activity of several enzymes. Khatri and Chenulu (1970) studied the changes in the peroxidase enzyme activity in leaves of resistant and susceptible cowpea varieties. Peroxidase activity increased in both resistant and susceptible varieties, but was higher in susceptible variety. Catalase activity decreased in the leaves of resistant cultivar and increased in the leaves of susceptible variety. Same trend was observed in tomato plants infected by tobacco mosaic virus and Potato virus X and in cucumber plants infected with cucumis virus 2 and 2A (cucumber green mottle and cucumber acuba mosaic viruses) (Serova et al., 1972). Batra and Kuhn (1975) found that when primary leaves of hypersensitive soybean plants were infected with cowpea chlorotic mottle virus, the enzymes polyphenoloxidase and peroxidase increased 2-3 times. They found that the increases were concomitant with the development of acquired resistance. Lee et al. (1975) while investigating the aspartate transcarbamylase activity in healthy and virus-infected cowpea and soybean leaves found that the enzyme activity was increased by nearly four times.

Several investigators have studied the activity of three common enzymes, peroxidase, polyphenoloxidase and ribonuclease, and have outlined quantitative changes in activity, before, during and after virus induced necrosis (Loebenstein, 1972; van Loon, 1982; Wagih and Coutts, 1982a).

Qualitative alterations that occur in enzymes after hypersensitive virus infection were also well documented in peroxidase (Bates and Chant, 1970; van Loon and Geelen, 1971), polyphenoloxidase (van Loon and Geelen, 1971; Sheen and Diachun, 1978) and in RNAse (Wyen et al., 1972).

Wagih and Coutts (1982b) found that in tobacco necrosis virus infected cowpea and cucumber, showed alterations in soluble protein and was accompanied by an increase in the amounts of extractable peroxidase, polyphenoloxidase and RNAse activity.

2.8.8 Phenol metabolism

Cowpea mosaic virus infected seeds contained a higher percentage of phenols than healthy seeds (Padma et al., 1976). Ramiah (1978) found that there was no difference in phenolics content between healthy and inoculated leaves of two cultivars MS 9804 and Co.1. He found that in variety Co.2 the inoculated leaves had higher content of phenolics than that of healthy

leaves only at 40 days after inoculation. Ando et al. (1984) reported that fungitoxic phenolic compounds were released from cucumber mosaic virus infected cowpea protoplast. Sastry and Nayudu (1988) could find higher quantities of phenolic compounds in hypersensitive cowpea primary leaves infected with tobacco ringspot nepovirus. Kato et al. (1993) extracted and characterised two phenolic compounds from cowpea leaves infected with cucumber mosaic virus. They identified the two phenolic compounds (isoflavonoids) as coumestrol and dalbergioidin.

## 2.9 MANAGEMENT OF CAMV

### 2.9.1 Effect of chemicals

Antiphytoviral chemicals are now widely used for the mangement of virus diseases. Several promising antiphytoviral substances have recently been described.

Woods and Agrios (1974) reported that solutions of enzymatically oxidized 1-$\beta$-3,4-dihydroxy phenylalanine (L-DOPA), chlorogenic acid and catechol reduced the infectivity of cowpea chlorotic mottle virus ribonucleic acid (CCMV-RNA). They found that the CCMV-RNA infectivity was reduced nearly by 70 per cent. Sharma and Varma (1975a) observed that soaking seeds in malic hydrazide at 40, 100 and 400 ppm for 90 minutes, 2-thiouracil at 500 or 700 ppm for 60 minutes, NAA at 40 ppm for 240 minutes and

teepol at 5 to 10 per cent for 240 minutes eliminated seed transmission of cowpea banding mosaic disease without affecting germination. Verma and Awasthi (1978) found that thiosemicarbazone derivatives inhibited gomphrena mosaic virus in cowpea plants. They also found that the antiviral activity varied from host to host and was dependent on time of application. Prakash and Joshi (1979) obtained a maximum inhibition of cowpea banding mosaic virus by 92 per cent when six sprays of gallic acid were applied after inoculation. They observed a maximum increase in the incubation period when roots were dipped in salicylic acid, 24 h before inoculation. Rao and Raychaudhuri (1980) reported that 1000 ppm sodium dodecyl sulphate (SDS) could completely inhibit cowpea mosaic virus infection.

Cowpea vein banding mosaic virus was inhibited 60 per cent by molybdophosphoric acid (peroxy), 56 per cent molybdo arsenic acid and 41 per cent by molybdo ceric acid (Agarwal et al., 1981). Prakash and Sadruddin (1983) observed that the inactivating capacity of phenolic acids was affected by pH levels. The infectivity was reduced more at acidic levels. They found that salicylic acid gave 69.51, 47.97 and 2.91 per cent inhibition of cowpea banding mosaic virus infectivity at pH 6.4, 6.7 and 7.3 respectively. They also observed that protocatechuic acid was only inhibitory and gallic acid was more effective at acidic levels. Mancino and Agrios (1984) found that

CCMV symptoms were suppressed when cowpea plants were sprayed with ribavirin, amantadine, formycin and methisazone but not Bavistin (carbendazim). Dawson (1984) reported that adenine arabinoside, ribavirin, (S) 9-(2,3-dihydroxypropyl) adenine and 5 azacytidine effectively inhibited CCMV. Lozoya and Dawson (1985) also got the same results with TMV and CCMV on mechanically inoculated or systemically infected plants. Sharma and Varma (1986a) while conducting studies on the effect of chemicals on infectivity and transmission of cowpea banding mosaic virus, found that indole acetic acid (IAA) and 2-thiouracil were more effective than other chemicals tested in delaying symptom development on cowpea, when sprayed one day before inoculation with the virus. They also observed that post-inoculation sprays of gibberellic acid, 2-thiouracil and indole butyric acid reduced seed transmission. Rao et al. (1988) could induce resistance in non-hypersensitive cowpea cultivars against legume viruses by spraying 100 µg/ml 12-tungsto zincic (II) acid solution (TZA). Bauer et al. (1993) observed that the chemical 1-(α -carboxyalkyl)-4,5-dimethylimidazol-3-oxides had antiphyto-viral activity against systemically infected red clover mottle virus (RCMV) and alfalfa mosaic virus (AMV). They also found that the virus concentration was reduced in systemically infected hosts for more than six weeks after application of the compound.

2.9.2 Plant extracts

Extracts from higher plants, which show high degree of resistance against pathogens in nature contain inhibitory substances showing antiviral activity against plant viruses. Several plants were known to contain potent inhibitors, which interfere with virus infection and multiplication.

Shukla and Joshi (1980) reported that the leaf extracts of *Capsicum annum* and *Datura stramonium* gave maximum inhibition of sugarcane mosaic potyvirus, which ranged from 10-75 per cent. Verma and Kumar (1980) found that foliar application of *Mirabilis jalapa* leaf extracts caused marked suppression of disease symptoms in legumes infected with viruses. They also observed that the population of aphids on treated plants were much lower than in control plants. Bose *et al*. (1983) observed that the leaf extracts of *Adenocalymma allicea* contained an inhibitor of bean common mosaic virus infection. They found that it was more effective when used with virus or when sprayed before inoculation. They also observed the extract prevented the acquisition of the virus by aphids. Verma *et al*. (1985) reported that the infection of legume viruses were suppressed by aqueous partially clarified leaf extracts of *Clerodendrum fragrans* and *Aerva songuinolenta* and root extracts of *Boerhaavia diffusa*. They found that the extracts from *C.fragrans* reduced infection by

60 per cent, whereas the other extracts only delayed the appearance of disease symptoms. Rao et al. (1986) found that essential oil from Ageratum conyzoides, Callistemon lanceolatus, Carum copticum, Ocimum sanctum and Peperomia pellucida had inhibitory activity against cowpea mosaic virus, mung bean mosaic virus, bean common mosaic virus and southern bean mosaic virus. They also observed that oil from O.sanctum at 3000 ppm gave the best inhibition ranging from 88 to 93 per cent. The antiviral activity of both leaf and root extracts of Phyllanthus fraternus was described by Saigopal et al. (1986), against virus diseases of cowpea. They found that mixing the inhibitor with the virus inoculum was more effective than application 24 h before or after inoculation.

Leaf and bark extracts of Azadiracta indica inhibited infection of C.amaranticolor by cowpea mosaic virus (Singh et al., 1988). They also observed that the inhibitor was heat stable and remained active even after 25 days of storage. Duarte et al. (1990) showed the inhibitory action of leaf extracts from Bougainvillea spectabilis, M.jalapa and Phytolaca americana against potyvirus symptom development. A non-hydroxylated alkaloid extracted from plants of Crinum augustum was inhibitory to potyvirus (Fahmy and Mohamed, 1990). Chen et al. (1991) reported that pokeweed antiviral protein (PAP) from P.americana could inhibit potyvirus. An inhibition of 68 per cent was obtained at

25 ng/ml of purified PAP, compared with the unprotected control. They also found that PAP partially prevented the transmission of potyvirus by aphids. Molina and Leon (1991) observed the antiviral activity of leaf extracts of *B.spectabilis*, *C.annum*, *Datura metel* and *D.stramonium* on potyvirus. They found that the extracts from *D.stramonium* and *B.spectabilis* were highly inhibitory when applied after inoculation. Sadasivam *et al*. (1991) reported antiphytoviral activity of *B.diffusa*, *B.spectabilis*, *Clerodendron aculeatum* and sorghum against cowpea aphid-borne mosaic virus. They found that all the plant extracts were effective upto 20 days of storage. Patel and Patel (1993) observed a 100 per cent inhibition of cowpea chlorotic mottle virus, when aqueous leaf extracts of *Clerodendrum inerme*, *Parkinsonia aculeata* and *Ipomea carnea* were mixed with the virus inoculum.

### 2.9.3 Aphid management

#### 2.9.3.1 Chemicals

Chemical control of aphid-borne virus diseases in crops relies on insecticides killing the vector fast enough to prevent secondary spread. Sharma and Varma (1974) while investigating the effect of systemic insecticides on virus infection in cowpea found that the chemicals phorate, aldicarb and dimethoate effectively reduced the incidence of the viruses. They also observed that phorate treatment increased the yield by 13 per cent. The

fast-acting synthetic pyrethroids (Elliott et al., 1978) may also control primary infection even by aphids carrying non-persistent viruses. Gibson et al. (1982) reported that the pyrethroid, deltamethrin restricts transmission of persistent, semi-persistent and perhaps more importantly of non-persistent viruses in the glasshouse and has potential for doing the same in the field. Gibson (1983) found that the synthetic pyrethroids inhibited the spread of potato viruses by aphids.

A mixture of the pyrethroid WL85871 (an enriched form of cypermethrin) and the mineral oil SC811 intoxicated adult apterae of an insecticide-susceptible clone of the peach-potato aphid, M.persicae at a similar rate to a treatment containing only WL 85871, but the mixture killed more (Gibson and Rice, 1986). They found that a mixture of the pyrethroids delta-methrin, cypermethrin and PP321 with SC811 or a mixture of WL 85871 with the mineral oil Bayol52 also decreased acquisition of PVY and another non-persistently transmitted virus viz., beet mosaic virus. Sulyo (1987) used a chemical Supracide (Methida-thion) for the control of CAMV and its vector A.craccivora on yard long bean (Vigna unguiculata). Roberts et al. (1993) found that the sprays of synthetic pyrethroids deltamethrin and lambda-cyhalothrin, eight days apart prevented aphid colonisation and within crop spread of virus by the colonising A.craccivora. They

also reported that neither of the pyrethroids used could prevent the initial introduction of CAMV into the cowpea crop.

2.9.3.2 Oils

Oils, which interfere with transmission, are also used to protect against non-persistent viruses. Hein (1972a,b) reported the inhibition of mineral and plant oils of mechanically transmitted viruses and prevented the aphid transmission. Dubey and Nene (1975) found that the viruliferous aphids lost the virus while probing on cowpea plants sprayed with emulsion of castor (2.5%), light paraffin (3, 3.5 and 4%) and emulsifiable oils (2.5 and 3%). They also observed that the spread of CAMV by aphids from sprayed infected to sprayed healthy was appreciably delayed due to oil sprays. Gibson and Rice (1986) reported that mineral oils in combination with synthetic pyrethroids effectively controlled PVY and decreased the acquisition of non-persistently transmitted viruses. Sulyo (1987) found that a mineral oil (Mesran 2E) and a plant oil (Maize oil) sprayed alone and in combination with supracide (Methidathion) effectively controlled CAMV and its vector A.craccivora on yard long bean.

2.9.3.3. Host plant resistance

2.9.3.3.1 Virus resistance

Ladipo and Allen (1979) identified disease resistant lines of cowpea germplasm against CAMV under glasshouse condition. They could screen 52 lines immune, 6 tolerant and the rest with mixed reaction from the germplasm. They found that the line TVu 1948 showed combined resistance to CAMV and *A.craccivora*, with relatively broad spectrum resistance to southern bean mosaic virus (SBMV). Twelve lines (TVu 79, 113, 310, 393, 445, 493, 1061, 1888, 1985, 1987, 2155 and 6365) showed combined resistance to some isolates of CAMV, with resistance to SBMV and CPMV (Williams, 1977; Ladipo and Allen, 1979). They also observed that the lines TVu 1185 and 1330 have broad spectrum resistance to CAMV and CPMV. Out of 23 varieties screened only C 288 was immune to CAMV and bean yellow mosaic virus. Sharma and Varma (1981) screened 26 cowpea cultivars by artificial inoculation and found that only three lines, Folden Type Grey, EM 2543 and EC 19739 were resistant to cowpea banding mosaic virus. Doraiswamy *et al*. (1983) reported that the cowpea line MS 9804 was resistant to CAMV. Collins *et al*. (1985) could identify five lines *viz*., Brown Crowder, Mangolia Blackeye, Mississipi Silver, Mississipi Purple and Worthmore, which showed promising levels of resistance to BICMV. Haque *et al*. (1985) found that the cowpea varieties CG 7, CG 28, Gomti, KBC 1, S 488,

CG 104 and CG 112 were resistant to cowpea mosaic virus. Ponte *et al.* (1985) could identify that a single variety, Otilia was highly resistant to CAMV under glasshouse inoculation trials. Rossel *et al.* (1985) found that the germplasm accessions TVu 410 and TVu 1948 appeared to be good virus-resistance donors. They also reported that advanced breeding lines, IT 82D-885, IT 82D-889 and IT 82E-60 were resistant to all eight isolates of CAMV tested.

Singh and Ntare (1985) and Thottappilly and Rossel (1985) reviewed the development of cowpea lines with multiple resistance to different viruses infecting cowpeas. Ponz *et al.* (1988) found that cowpea cultivar Arlington was immune to cowpea mosaic virus. They reported that cowpea mosaic virus was controlled by a single dominant gene in crosses to the susceptible line Blackeye 5. Quindere and Barreto (1988) could identify seven genotypes out of 81 evaluated were resistant to CAMV. Mih *et al.* (1991) reported that the line TVu 15656 was highly resistant to CAMV. Several improved cowpea lines with multiple resistance have been developed in International Institute of Tropical Agriculture (IITA) in Nigeria (Singh *et al.*, 1992). They reported the following varieties were resistant to CAMV: IT 81D-1137, IT 82E-16, IT 82D-889, IT 83S-818, IT 83D-442, IT 84D-449, IT 85F-867-5 and IT 85F-2687.

#### 2.9.3.3.2 Aphid resistance

The cowpea aphid is a seedling pest, but it also infests flowers and green pods on older plants. High levels of resistance to this pest have been identified. Several lines, including TVu 310, TVu $408P_2$, TVu 801 and TVu 3000 were highly resistant to aphid infestation (Singh, 1977; Singh and Jackai, 1985). Resistance to the cowpea aphid has been successfully incorporated into several breeding lines, eliminating the need for other control measures (Singh *et al.*, 1992).

## 2.10 *IN VITRO* CALLUS CULTURE AND REGENERATION

*In vitro* plant cell and tissue culture techniques are powerful tools for the development of new cultivars.

Gowda and Satyan (1984) reported callus induction and regeneration of whole plants from hypocotyls of cowpea. Ramachandran and Mishra (1989) observed that callus cultures of leaves of cowpea, supported virus concentration higher than those in greenhouse grown plants. They also found that there was no differentiation. Chema and Bawa (1992) observed that embryogenic callus could be obtained only with 2,4-D. They found that NAA favoured rhizogenesis in all the explants, while shoot regeneration was only observed from the stem explant (with apex) when a

kinetin-supplemented Murashige and Skoog medium was used. Rathor et al. (1994) reported that shoot tips of 4 to 7 day old seedlings of V.unguiculata cv. RC-8 could be cultured on agar solidified MS medium, supplemented with different auxins (2,4-D, IAA and NAA) in different combination with cytokinin (BAP and Kn). They could also find that multiple shoot buds were induced on media supplemented either with a cytokinin singly or in combination with NAA, 2,4-D or IAA.

## 2.11 SERODIAGNOSIS OF CAMV

Govindaswamy et al. (1970) found that the antiserum produced specific to cowpea mosaic virus, gave positive reaction with the virus, while negative reaction was observed in Dolichos ringspot virus, safflower mosaic virus and tobacco mosaic virus in the slide agglutination test. They also got the same results in agar gel diffusion test. Reciprocal double immunodiffusion tests with SDS-treated antigen showed that BICMV was serologically related to but distinct from other potyvirus tested (Lima et al., 1979). They found that the antiserum for BICMV also reacted specifically with CAMV forming a distinct spur which extended past the heterologous reaction. Lin et al. (1981) identified elongated fluxuous rods of cowpea green vein banding and blackeye cowpea mosaic viruses by immunodiffusion in agar gel containing SDS. Taiwo and Gonsalves (1982) compared the

isolates of CAMV and BICMV using Enzyme-linked immunosorbent assay (ELISA), immune electron microscopy (IEM),and immuno-diffusion in sodium dodecyl sulphate. They found that the anti-sera to isolates of the CAMV group did not detectably react to isolates of the BICMV group. Dijkstra et al. (1987) could not assign any of the five isolates categorically to either BICMV or CAMV on the basis of immunodiffusion tests and in ELISA. Mali et al. (1989) used serological tests consisting of slide agglutination, ring interface precipitin and Ouchterlony double diffusion for the diagnosis of six cowpea seed-borne viruses in India. Bashir and Hampton (1993) reported that the seed-borne nature of CAMV and BICMV could be detected using direct antigen coating (DAC) or double antibody sandwich (DAS) ELISA from the desiccated samples. Gillaspie et al. (1993) reported that BICMV potyvirus was seed-borne and could be detected by DAS-ELISA. They found that the virus occurred in cotyledons and embryo axes and very little in or on the testae. Huguenot et al. (1993) reported that CAMV and BICMV were two different potyviruses. They conducted a serological study including reference isolate followed by further characterization in different hosts resulted in the separation of the potyviruses into two distinct serogroups, using monoclonal and polyclonal antibodies. Nain et al. (1994) also used Ouchterlony double diffusion tests for identifying eight viruses commonly occurring in cowpea in north India.

# *MATERIALS AND METHODS*

# CHAPTER 3

# MATERIALS AND METHODS

## 3.1 MAINTENANCE OF CAMV CULTURE

The CAMV isolate was obtained from diseased plants of cowpea (*Vigna unguiculata* spp. *unguiculata*) cv. CO 4 raised from seeds supplied from the Pulses Breeding Station, TNAU, Coimbatore. The virus culture was maintained on cowpea cv. CO 4 by successive transfers by mechanical inoculation using 0.1M phosphate buffer pH 7.5 (16 ml of 0.1M monobasic potassium phosphate + 84 ml of 0.1M dibasic potassium phosphate) at periodic intervals of 4-6 weeks under insect proof glasshouse conditions maintained at 24-32 C. Test plants showing typical mosaic symptom were used as the initial source of inoculum for virus propagation. The systemically infected plants were maintained in earthenware pots of 30 cm diameter in a mixture of soil:sand:dry cowdung (2:1:1). Plants were fertilized with a 20-20-20 (N-P-K) solution at weekly intervals.

## 3.2 MASS CULTURE OF VECTORS

Colonies of aphids were established from apterous single clonal line of each species were used in all tests. Respective host plants for each species were infested with a single aphid and maintained in separate cages in a greenhouse.

*A.craccivora* colonies were reared on healthy *Dolichos lablab* L., *A.gossypii* on *Gossypium hirsutum* L., and *M.persicae* on *Capsicum annum* L.

The *Bemisia tabaci* culture was maintained on *Solanum melongena* L., Bean leaf beetles, *Cerotoma trifurcata*, were collected from field and fed on *Phaseolus vulgaris* L.

Plants of each species were grown from seeds in an insect- and virus-free greenhouse and used to replace plants in the vector colonies on a regular basis, thus assuring vigorous vectors for transmission trials.

3.3. HANDLING OF VECTORS

The different aphid species were removed one by one by means of a pointed artist's brush with moistened tip to make the insect adhere to the brush.

The whiteflies were handled by using an aspirator consisting of a small glass bottle closed with a two-hole rubber stopper. A small straight glass tube was inserted through one of the holes. One end of this tube was connected to a rubber tubing which serves as a mouthpiece, the other end was covered with a small piece of screen. A slightly longer glass tube of desired

shape was inserted into the other hole. The whiteflies were gently sucked into the glass bottle and were subsequently used either for acquisition access feeding or inoculation access feeding (Green, 1991).

For virus transmission trials with beetles, single beetle was fed on virus-infected plants and then transferred to young test plants.

## 3.4 CAGES FOR TRANSMISSION STUDIES

For transmission studies, plastic cylinder plant cages of various sizes were made. Cellulose nitrate plastic sheets were used for making cages. The top of the cage was covered with cheese cloth. A small hole was made at the middle portion of the cage to release the insects inside the cage. If the plants under test, were small enough to be accommodated inside the plastic cylinder, the plants were covered with plastic cylinder cages and the bottom of the cages were pressed into the soil of the pot. If the plants were big, a twig or leaf was inserted into the plastic cylinder cages and the open end of the cages were plugged with cotton.

## 3.5 TRANSMISSION

### 3.5.1 Mechanical transmission

Mechanical transmission studies were made by sap extracted by triturating infected leaves of cowpea with a mortar and pestle in cold 0.1M phosphate buffer, pH 7.5 (about 1 g of leaf tissue to 5 ml of buffer). Test plants were inoculated by conventional leaf rub method with a cotton swab moistened with the inoculum or using forefinger, by gently rubbing on the upper surface of the fully opened cotyledonary leaves of 10-day old healthy cowpea plants (Takahashi, 1956; Kado, 1972). Carborundum powder (600 mesh) was used as an abrasive. Care was taken not to cause excess injury to the leaves during inoculation. Soon after the inoculations, the cotyledonary leaves were washed with distilled water using a wash bottle. Ten plants each, were inoculated for every experiment and an equal number of buffer inoculated plants were kept as control. The experiments were done twice and the plants were kept under observation in insect proof glasshouse conditions. The plants that did not show any symptoms after 4-6 weeks were back-indexed on *Chenopodium amaranticolor* for the recovery of virus.

### 3.5.2 Seed transmission

For seed transmission tests, seeds collected from virus infected and artificially inoculated plants of cowpea cultivars were grown in earthen pots containing steam-sterilized soil, sand and compost (2:1:1) mixture and maintained in an insect proof glasshouse. Seed-borne infection was determined by symptoms on the primary and first trifoliate true leaves. The level of seed transmission was determined by counting diseased plants from total population and expressed as per cent infection.

### 3.5.3 Insect transmission

Aphid transmission tests from cowpea to cowpea were determined by using *A.craccivora*, *A.gossypii* and *M.persicae*. About 10 non-viruliferous apterous aphids were starved for 1 h in glass vials and placed on leaves of CAMV infected young seedlings using a moistened pointed artist's brush. Aphids were permitted an acquisition access period of 30 minutes and were transferred to healthy cowpea seedlings for 4 h inoculation feeding period. Aphids were later killed by a spray of 0.05 per cent dimethoate insecticide, and the plants were maintained in an insect proof glasshouse for two weeks before indexing for virus infection.

For whitefly transmission, about 50 non-viruliferous _B.tabaci_ were starved for 1 h in the glass bottle of the aspirator and released onto caged infected cowpea seedlings. After an acquisition access period of 24 h the whiteflies were transferred onto caged healthy cowpea plants. The whiteflies were given an inoculation access period of 24 h and killed by a spray of 0.05 per cent dimethoate. The plants were kept in insect proof glasshouse for two weeks and observed for disease symptoms.

For virus transmission trials with bean leaf beetle (_Cerotoma trifurcata_), single beetle was fed on virus-infected cowpea plants for 24 h and then transferred to young test plants for a 24 h inoculation feeding period. Test plants were grown in the greenhouse for 2 weeks and then tested for virus infection (Gergerich _et al_., 1991).

### 3.6 VIRUS-VECTOR RELATIONSHIP

This experiment was conducted by using _A.craccivora_ which was found to be the most efficient vector. Non-viruliferous aphid colonies were maintained on suitable hosts in an insect rearing cage. In all the inoculation trials only fully grown apterous aphids were used. In the case of short feeding periods of less than 5 minutes the individual aphid was watched

through a magnifying lens and the time of feeding was determined with the help of a stop watch after the aphids had settled down to feed.

Preliminary investigations on virus-vector relationship were conducted under insect proof glasshouse conditions to standardize the minimum acquisition feeding period, inoculation access period, preacquisition and preinoculation starvation, number of aphids required for transmission and persistence of the virus in the vector.

### 3.6.1 Effect of acquisition access period

A large number of non-viruliferous aphids were collected and were given a preacquisition fasting of 1 h. Batches of 5 aphids, each were given acquisition feeding of 1, 5, 10, 20 and 30 minutes and 1, 3, 6 and 24 h on diseased source before transferring them to healthy cowpea plants. The aphids were then allowed to remain for 24 h on the test plants and were killed thereafter by spraying 0.05 per cent dimethoate.

### 3.6.2 Effect of inoculation access period

Non-viruliferous aphids were given 1 h preacquisition starvation and an acquisition feeding of 30 minutes. Then the

viruliferous aphids were transferred in batches of 5 to individual healthy test plants. Each batch was given separate inoculation feeding periods of 1, 5, 10, 15 and 30 minutes and 1, 6, 12 and 24 h. The aphids were killed after specific inoculation feeding period by spraying 0.05 per cent dimethoate.

### 3.6.3 Influence of starvation before acquisition and inoculation access periods

#### 3.6.3.1 Preacquisition starvation

A large number of aphids were starved for different periods such as 30 minutes, 1, 2, 3, 4, 5 and 6 h and batches of 5 aphids from each category were given an acquisition access period of 30 minutes on diseased plants before allowing them to feed on the healthy test plants. After 2 h the test plants were sprayed with insecticide to kill the vectors. The controls with equal number of aphids were maintained without preacquisition starvation.

#### 3.6.3.2 Preinoculation starvation

Non-viruliferous aphids were starved for 1 h and given an acquisition access period of 30 minutes. These viruliferous aphids were then starved again in batches of 5 for different periods such as 30 minutes, 1, 2, 3, 4, 5 and 6 h. Groups of 5 aphids from each of these categories were transferred to healthy

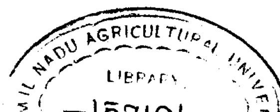
test plants and given 2 h inoculation access before they were killed with 0.05 per cent dimethoate.

### 3.6.4 Relation of number of viruliferous aphids for CAMV transmission

Single aphid as well as aphids in groups of 3, 5, 10, 15 and 20 were collected from a non-viruliferous colony from the rearing cage and were starved for 1 h. These aphids were made viruliferous by feeding them on diseased cowpea plants. After an acquisition feeding period of 30 minutes, the aphids were transferred to healthy test plants and were allowed to feed for 2 h. The aphids were then killed by spraying 0.05 per cent dimethoate.

### 3.6.5 Persistence of CAMV in *A.craccivora*

The experiments were conducted with viruliferous insects, which were transferred in succession to a series of healthy cowpea plants after giving a definite inoculation feeding period on each plant. For this experiment groups of aphids were starved for 1 h and fed on diseased cowpea plants for 30 minutes to make them viruliferous. Groups of 5 aphids were then transferred in succession to a series of five healthy plants transferring the insects after a definite interval. The



different feeding intervals allowed in different series were 30 minutes, 1, 1½, 2, 2½ and 3 h. The aphids were killed from the fifth plant of the different series using 0.05 per cent dimethoate. The experiment was done three times.

3.7 HOST RANGE OF THE VIRUS

To determine the host range of CAMV , 48 plant species belonging to 11 families were tested for their susceptibility by artificial inoculation (mechanical inoculation). The plants which did not show symptoms after four weeks were indexed by back inoculation to C.amaranticolor to find whether they were symptomless carriers of the virus.

3.8 ANATOMICAL STUDIES

In order to study the nature of damage caused by the virus in plant tissues, histopathological studies were undertaken. Specimens from apical meristem and leaves of healthy and diseased cowpea plants were analysed.

The fixation, dehydration, infiltration, embedding, sectioning, staining and mounting were done as per the procedure described by Johansen (1940).

### 3.8.1 Fixation and dehydration

The materials were fixed in Formalin-Aceto-Alcohol (FAA) solution for 24 h.

FAA solution

| | | | |
|---|---|---|---|
| Formaldehyde (40%) | ... | 10 | ml |
| Acetic acid (glacial) | ... | 5 | ml |
| Ethyl alcohol (70%) | ... | 85 | ml |

The fixed materials were dehydrated in tertiary butyl alcohol (TBA) series as follows:

| Series | Per cent concentration | TBA proportion, ml | Ethyl alcohol, ml | Distilled water, ml | Time, h |
|---|---|---|---|---|---|
| 1 | 60 | 10 | 50 | 40 | 1 |
| 2 | 70 | 20 | 50 | 30 | 1 |
| 3 | 85 | 35 | 50 | 15 | 2 |
| 4 | 100 | 50 | 50 | - | 2 |
| 5 | 100 | 75 | 25 | - | 2 |

The materials were further put in pure TBA overnight for 18 h and then again in fresh solution of pure TBA twice for periods of 1 h each.

### 3.8.2 Infiltration and embedding

Equal quantities of TBA and liquid paraffin were mixed and the plant materials were kept in it for 1 h. The materials along with the liquid paraffin, TBA and some amount of melted paraffin wax were then kept in an oven maintained at 60 C for 12 h. The melted wax was decanted and fresh melted was again added. This process was done 4-6 times at 2 h intervals.

Paper boats were made and a thin layer of glycerin was coated on the surface. The specimens were then arranged in the boats and the melted paraffin wax was poured into the boats. On cooling, the materials were embedded in the paraffin wax.

### 3.8.3 Sectioning and mounting

The embedded materials were sectioned using a Senior Rotary Microtome (Weswox) at 5 um thickness. the sections were then deparaffinised in xylene for 5 minutes, rinsed with 90 per cent ethyl alcohol for 2 minutes and then in running tap water for 2 minutes. The sections were then stained in Heidenhain's iron-alum-haematoxylin and mounted in DPX. The slides were then examined microscopically and photographed using Nikon Optiphot microscope system.

## 3.9 DETECTION OF CAMV

Early diagnosis of diseased plants even before appearance of visible symptoms will be helpful in management of the disease. Attempts were therefore made to develop reliable methods of diagnosis of the infected plants.

### 3.9.1 Detection of CAMV by direct antigen coating enzyme-linked immunosorbent assay (DAC-ELISA)

#### 3.9.1.1 Source of the virus (Antigen)

CAMV was propagated in cowpea plants (*V. unguiculata* spp. *unguiculata*) and the systemically infected leaves were used as virus source. Primary leaves of 5- to 7-day old cowpea seedlings were inoculated with the virus obtained by grinding infected leaf tissue in 0.1 M potassium phosphate buffer pH 7.5, containing 0.01 M EDTA (2 ml per g of tissue). The first trifoliate leaves showing typical mosaic were collected 15-18 days later and used as antigen source.

#### 3.9.1.2 Antibodies

Polyclonal antibodies ($Ab^R$) and Monoclonal antibodies (MAbs) of CAMV and BICMV were supplied by Dr.G.Thottappilly (International Institute of Tropical Agriculture, Ibadan, Nigeria). Southern bean mosaic virus, cowpea mottle virus, cucumber mosaic virus and cowpea mosaic virus polyclonals and

eight monoclonals, four each of CAMV and BICMV (5H5 CAMV-Fenkan, 1F5 CAMV Monguno, 6C10 CAMV 70.12, 7D9 CAMV-Nkeji; 15E6 BICMV 81.1, 12F9 BICMV, 10G5 BICMV 81.11, 16G5 BICMV 81.11) were used for serodiagnosis.

Alkaline phosphatase (ALP) conjugated goat antirabbit immunoglobulin (IgG) (Source: Sigma Immunochemicals, Product No.A 3687).

3.9.1.3 Reagents

a) For serological studies, crude extracts from infected plants were used. The leaf material was ground in phosphate buffered saline containing 0.05 per cent Tween 20, pH 7.4 (PBS-T), using a ratio of 1 g leaves/25 ml buffer.

b) Phosphate buffered saline-Tween (PBS-T, pH 7.4) washing solution:

| | | |
|---|---|---|
| Disodium hydrogen phosphate ($Na_2HPO_4.2H_2O$) | ... | 1.44 g |
| Potassium dihydrogen ortho phosphate ($KH_2PO_4$) | ... | 0.20 g |
| Potassium chloride (KCl) | ... | 0.20 g |
| Sodium chloride (NaCl) | ... | 8.00 g |

| | | | |
|---|---|---|---|
| Tween-20 | ... | 0.50 | ml |
| Distilled water | ... | 1.00 | l |

c) Antibody/conjugate dilution buffer (PBS-TPO)

| | | | |
|---|---|---|---|
| PBS-T | ... | 1.0 | l |
| PVP | ... | 20.0 | g |
| Ovalbumin | ... | 2.0 | g |

d) Substrate buffer, pH 9.8

| | | | |
|---|---|---|---|
| Diethanolamine | ... | 97.00 | ml |
| Distilled water | ... | 800.00 | ml |

The pH was adjusted to 9.8 with 1N HCl and the volume was made upto 1000 ml with distilled water. The substrate buffer was stored at room temperature to prevent its solidification in cold.

e) Substrate

Substrate was prepared freshly just before use by dissolving P-nitrophenyl phosphate (PNP) to a concentration of 1 mg/ml in diethanolamine substrate buffer.

f) Stop solution

| | | | |
|---|---|---|---|
| Sodium hydroxide (NaOH) | ... | 4.0 | g |
| Distilled water | ... | 100.0 | ml |

This was used for stopping the enzyme substrate reaction.

3.9.1.4 Procedure

The leaf material was ground in PBS-T using a ratio of 1 g leaves/25 ml buffer. The homogenate was centrifuged at 10,000 g for 10 min at 4°C. the antigen was then dispensed into wells (100 µl/well) of Nunc Immunological plates type II. The treatments were replicated twice. After incubation and washing the antigen was exposed to the primary antibodies ($10^{-4}$ dilution) and incubated. The alkaline phosphatase conjugated antirabbit immunoglobulin (100 µl) was added to each well at a dilution of 1 µg/ml with antibody dilution buffer. Then the substrate (p-nitrophenyl phosphate in diethanolamine buffer) was added to the wells (100 µl/well) and incubated for 1 h at 37 C. The reaction was stopped by adding 4 per cent NaOH. Suitable control wells were maintained using extracts of healthy plant and the antigen extraction buffer. The absorbance of the well content was read at 405 nm in an ELISA reader, Bio-Rad Model 3550 Microplate Reader (Huguenot *et al*., 1993).

### 3.9.2 Detection of CAMV by a modified indirect enzyme-linked immunosorbent assay on nitrocellulose membranes (NCM-ELISA)

NCM-ELISA was carried out to detect CAMV in sap extracts of infected cowpea leaves and body fluid of viruliferous aphids as per the method described by Lizarraga and Fernandez-Northcote (1989).

#### 3.9.2.1 Antigen

For the antigen from cowpea leaves the specimens were prepared as described in 3.9.1.1.

For the antigen from viruliferous vectors, aphids (*A. craccivora*) were collected from pure culture and maintained on healthy cowpea plants in the insect proof cages. The aphids were given 1 h preacquisition starvation and an acquisition access period of 1 h on CAMV infected cowpea plants. The aphids were collected after the completion of the acquisition access period for the assay.

#### 3.9.2.2 Antibody

Polyclonal antibodies ($Ab^R$) produced against CAMV in rabbit

ALP conjugated goat antirabbit IgG

### 3.9.2.3 Reagents

a) Washing buffers

i) Tris-buffered saline (TBS)

TBS consisted of 20 mM Tris (hydroxymethyl) amino-methane buffer, pH 7.4, containing 500 mM NaCl

ii) TBS-Tween (TTBS)

TTBS consisted of TBS with 0.05 per cent Tween-20

b) Buffer for antigen extraction and dilution

The buffer used for antigen extraction and dilution was TBS containing 0.01M Na-DIECA, 2 per cent PVP and 0.2 per cent Bovine serum albumin (BSA).

c) Buffer for antibody/conjugate dilution

Antibodies and enzyme conjugated antibodies were diluted in TBS containing 2 per cent PVP and 0.2 per cent BSA.

d) Blocking buffer

TBS containing 3 per cent BSA was used as blocking buffer.

e) Substrate buffer

Substrate was prepared in 0.1M Tris-base buffer adjusted to pH 9.5 with 5N HCl and 0.1M NaCl and 5 mM magnesium chloride ($MgCl_2$) were added.

f) Substrate solution

Substrate solution for colour development was prepared by dissolving 10 mg of p-nitro blue tetrazolium (NBT) in 30 ml of substrate buffer protected from light. Immediately before use, 5 mg of p-toluidine salt of 5-bromo-4 chloro-3-indolyl phosphate (BCIP) dissolved in 100 ul of N,N-dimethyl formamide was added in 10 µl drops to the NBT solution while the mixture was being shaken.

3.9.2.4 NCM-ELISA procedure

Infected leaf samples were thoroughly homogenized in antigen extraction buffer using chilled pestle and mortar. The homogenate was centrifuged at 10,000 rpm for 10 minutes at 0 C.

The supernatant was diluted in the same buffer to give antigen dilutiòn of 1:10, 1:50, 1:100 and 1:1000. Frozen viruliferous aphids were crushed in sterile Eppendorf tubes using ground glass rod at the rate of 1, 5, 10 and 20 insects per 10 µl of the antigen extraction buffer separately and centrifuged at 10,000 rpm for 10 min at 0 C.

Soaking, washing and incubation were carried out in sterile 9 cm diameter petri plates. A grid (1.5 x 1.5 cm) was drawn on the 7 x 6 cm NCM (pore size, 0.45 um) with pencil. The NCM was handled with forceps and immersed 5 min in sterile distilled water and then 5 minutes in TBS. Care was taken not to entrap air. Two sheets of Whatman No.1 filter paper were soaked for 10 min in TBS and placed on two dry Whatman No.1 filter papers. the NCM was placed on top and allowed to dry for 1-2 minutes.

Antigen solutions (10 µl) were dotted on each square and allowed to dry. The NCM was then transferred onto another dry filter paper for a further 2 h of drying.

The NCM was put in 15 ml of the blocking buffer for 1 h, then transferred into TBS for a brief wash. Then, 250 µl per spot of polyclonal rabbit antisera raised against CAMV (1:1000 dilution in antibody buffer) was added and the membrane was

incubated overnight in a sealed plastic plate placed on a gyratory shaker at 50 rpm. The NCM was rinsed very briefly in TTBS, then washed three times (10 min each) with TTBS 3 ml/$cm^2$ of membrane. Membranes were incubated 1 h with goat antirabbit IgG-alkaline phosphatase conjugates diluted 1:1000 in the antibody buffer, rinsed briefly with TTBS, and washed as described above. Colour development solution (250 µl per spot) was applied to the membranes and incubated for 1 h.

Membranes were washed three times with distilled water, placed on dry filter paper, and dried. Positive reactions appeared as bluish purple spots.

### 3.9.3 Electron microscopy (EM)

Electron microscopic detection of CAMV was conducted using concentrated virus preparation. The clarified preparation from the infected tissue extract has been found to be highly effective, since this clarified virus concentrate (CVC) provides cleaner preparation suitable for transmission electron microscopic screening. The standard procedure for obtaining a CVC from CAMV infected leaves is as follows:

i) Infected leaf tissue (1 g) was thoroughly homogenized in 2 ml of 0.1 M potassium phosphate buffer pH 7.5,

containing 0.01 M Na-DIECA, 0.1 per cent sodium sulphite and 2 per cent PVP. The homogenate was squeezed through cheese cloth.

ii) The filtrate was stirred with equal volume of n-butanol and chloroform mixture (1:1 v/v) for 10 minutes.

iii) The slurry was centrifuged at 10,000 rpm for 10 minutes and the pellet was discarded.

iv) To the supernatant, polyethylene glycole (PEG-6000) and sodium chloride (NaCl) was added to make 6 per cent and 0.125 M respectively and allowed to stand for 30 minutes under constant stirring.

v) The mixture was centrifuged at 10,000 rpm for 10 minutes.

vi) The supernatant was discarded and the tubes were inverted on a dry filter paper to remove all the trace of liquid fraction.

vii) The pellet was resuspended in 100 µl of the extraction buffer and centrifuged at low speed (5000 rpm) for 5 minutes.

viii) The pellet was discarded and the supernatant was used for coating grids for transmission electron microscopy (TEM) and Immunosorbent electron microscopy (ISEM).

3.9.3.1 TEM of CAMV

A drop of the CVC (30 µl) was placed on a piece of parafilm and a collodion coated copper grid (400 mesh) was floated on the drop of virus suspension, the film side down, for 5-10 minutes. The grid was then washed with 30 drops of distilled water followed by five drops of 2 per cent aqueous uranyl acetate and the grid was allowed to dry on a filter paper for a few minutes. Each treatment was replicated three times. The grids were then observed under Jeol-JEM 100 SX transmission electron microscope.

3.9.3.2 ISEM of CAMV

Collodion coated copper grids (400 mesh) were floated on 30 ul drops of antisera to CAMV, previously diluted to 1:50 with 0.1 M potassium phosphate buffer, pH 7.5 for 10 minutes. The grids were then rinsed with 20 drops of phosphate buffer and dried briefly on a filter paper. The antiserum coated grids were floated on 30 ul drops of virus suspension, placed on a piece of parafilm. It was then incubated for 1 h at 37 C in a humid petri

dish. The grids were rinsed again with 20 drops of buffer and then 5 drops of 2 per cent aqueous uranyl acetate. The grids were blotted dry of the staining solution by touching its edge on a piece of filter paper and left for air drying. The grids were then screened under Jeol-JEM 100 SX transmission electron microscope.

## 3.10 PHYSIOLOGY AND BIOCHEMISTRY OF VIRUS INFECTED PLANTS

### 3.10.1 Estimation of total chlorophyll

The total chlorophyll was estimated by the method detailed by Arnon (1949). One g of leaf samples (harvested from cowpea plants, 10, 20, 30 and 40 days after inoculation, along with its control), were homogenized in a clean mortar with a pestle by adding 20 ml of 80 per cent acetone. The homogenate was centrifuged at 5000 rpm for 5 minutes and the supernatant was transferred into a 100 ml volumetric flask. The residue was again homogenized with 20 ml acetone. The extraction was repeated three times with 80 per cent acetone till the residue was colourless. All the washings were collected in the volumetric flask. The volume was made upto 100 ml using 80 per cent acetone. The absorbance of the solution was read at 645 and 663 nm against the solvent (80% acetone) blank using Hitachi 200-20 spectrophotometer. The total chlorophyll content was calculated using the following equation and expressed as mg chlorophyll per g tissue.

$$\text{Total chlorophyll} = 20.2\ (A645) + 8.02\ (A663) \times \frac{V}{1000 \times W}$$

where,

A = absorbance at specific wavelength

V = final volume of chlorophyll extract in 80 per cent acetone

W = fresh weight of tissue extracted

3.10.2 Estimation of total carbohydrate

Total carbohydrate content in cowpea leaves was estimated by Anthrone method (Hedge and Hofreiter, 1962). One hundred mg of leaf samples (harvested from cowpea plants, 10, 20, 30 and 40 days after inoculation along with its respective controls) were weighed into boiling tubes and hydrolysed by keeping in a boiling water bath for 3 h with 5 ml of 2.5N HCl and cooled to room temperature. The hydrolysate was neutralised with solid sodium carbonate until the effervescence ceased. The volume was made upto 100 ml and centrifuged at 10,000 rpm for 10 minutes. To 0.5 ml of the supernatant, 0.5 ml of distilled water and 4 ml of anthrone reagent (200 mg anthrone in 100 ml of ice cold 95 per cent $H_2SO_4$) was added. The reaction mixture was heated for 8 minutes in a boiling water bath and cooled rapidly. The colour of the solution becomes green to dark green which was read at 630 nm using Hitachi-200-20 spectrophotometer. D-glucose was used as standard. Total carbohydrate content was expressed in terms of milligrams of glucose equivalent per g of leaf tissue on fresh weight basis.

### 3.10.3 Estimation of soluble protein

Total soluble protein content was estimated as per the procedure described by Bradford (1976).

The stock dye solution was prepared by dissolving 100 mg of coomasie brilliant blue G-250 in 50 ml of 95 per cent ethanol and 100 ml of concentrated O-phosphoric acid was added to it. The volume of the solution was made upto 200 ml with water and kept at 4° C. The working dye solution was prepared just before use by diluting the stock dye solution 5 times with water.

The protein extract was prepared by grinding 1 g of leaf sample in 5 ml of 0.1M sodium acetate buffer pH 4.7 in a chilled pestle and mortar. The homogenate was centrifuged at 10,000 rpm for 10 minutes at 4° C and the supernatant was used for the assay of soluble protein.

The estimation was done by adding 0.5 ml of the protein extract, 0.5 ml of water and 5 ml of the working dye solution in a test tube and incubated for 5 minutes at room temperature. The blank consisted of 1 ml water and 5 ml of the working dye solution. The intensity of the blue colour was recorded at 595 nm using Hitachi 200-20 spectrophotometer. Bovine serum albumin was used as the protein standard. The soluble protein content

was expressed as µg albumin equivalent of soluble protein per g on fresh weight basis.

### 3.10.4 Electrophoretic separation of soluble proteins by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Electrophoretic separation of soluble proteins of seeds and leaves were carried out as per the method described by Laemmli (1970).

#### 3.10.4.1 Electrophoretic analysis of seed proteins

Seed samples of susceptible (from healthy and diseased) and resistant plants were taken for the analysis. One hundred mg of powdered samples were homogenized in 1.5 ml of cold phosphate buffer (pH 7.0) at 4° C. The homogenate was filtered through cheese cloth and centrifuged at 10,000 rpm for 20 minutes at 4° C. The supernatant was used for SDS-PAGE. The extracts were loaded such that each well contained 50 µg of proteins. The protein content of the samples were estimated by the method described by Bradford (1976).

#### 3.10.4.2 Electrophoretic analysis of PR-proeins

Leaf samples of susceptible and resistant cowpea plants (healthy, inoculated and sprayed with AVPs) were used for the

analysis of PR-proteins. Samples of young cowpea leaves of 200 mg were ground in a chilled mortar and pestle with 1.5 ml of ice cold citrate-phosphate buffer (pH 2.8) containing 84 mM citric acid, 32 mM disodium hydrogen phosphate, 14 mM 2-mercaptoethanol and 5 mM ascorbic acid. The homogenates were filtered through a double layer of cheese cloth and the filtrates was centrifuged at 10,000 rpm for 20 minutes at 4°C and the supernatants were used for SDS-PAGE.

The protein extracts were precipitated with 5 volumes of ice cold acetone by keeping it in freezer (0°C) for 30 minutes. The precipitated protein were pelleted by centrifugation at 10,000 rpm for 15 minutes at 4°C. The pellets were dissolved in sample buffer and vortexed. The vortexed samples were boiled at 95°C for 4 min to ensure complete reaction between proteins and SDS, and cooled rapidly in ice water. These samples were used for SDS-PAGE. The protein concentration was adjusted in each sample to a strength of 100 µg of protein in 100 µl of sample buffer. In each well 100 µl of the samples were loaded.

Reagent

a) Acrylamide stock (30%)

| | | | |
|---|---|---|---|
| Acrylamide | ... | 29.2 | g |
| Bis-acrylamide | ... | 0.8 | g |
| Double distilled water | ... | 100.0 | ml |

b) Separating (resolving) gel buffer stock (1.5 M Tris-HCl pH 8.8)

Tris-base (18.15 g) was dissolved in approximately 50 ml of double distilled water. The pH was adjusted to 8.8 with 6N HCl and made up the volume to 100 ml with double distilled water and stored at 4°C.

c) Stacking gel buffer stock (0.5M Tris-HCl, pH 6.8)

Tris-base (6.0 g) was dissolved in approximately 60 ml of double distilled water and adjusted the pH to 6.8 with 6N HCl and the volume was made upto 100 ml with double distilled water and stored at 4°C.

d) Polymerising agents

Ammonium persulphate ... 10 per cent
(0.5 g in 5 ml double distilled water) prepared freshly before use.

TEMED - fresh from the refrigerator

e) Electrode buffer, pH 8.3

| | | | |
|---|---|---|---|
| Tris-base/HCl | ... | 6.0 | g |
| Glycine | ... | 28.8 | g |
| SDS | ... | 2.0 | g |
| Double distilled water to | ... | 2.0 | l |

f) Sample buffer (SDS-reducing buffer)

| | | | |
|---|---|---|---|
| Double distilled water | ... | 2.6 | ml |
| 0.5M Tris-HCl, pH 6.8 | ... | 1.0 | ml |
| 2-Mercaptoethanol | ... | 0.8 | ml |
| Glycerol | ... | 1.6 | ml |
| 20% (W/V) SDS | ... | 1.6 | ml |
| 0.5% Bromophenol blue (Optional) | ... | 0.4 | ml |

g) Staining solution

| | | | |
|---|---|---|---|
| Coomassie brilliant blue R 250 | ... | 0.1 | g |
| Methanol | ... | 40.0 | ml |
| Glacial acetic acid | ... | 10.0 | ml |
| Double distilled water | ... | 50.0 | ml |

h) Destaining solution

As above without coomassie brilliant blue

<u>Procedure</u>

Separating gel was first casted followed by the stacking gel by mixing the various solutions as indicated below:

a) Preparation of separating gel (12%)

| | | | |
|---|---|---|---|
| Double distilled water | ... | 6.7 | ml |
| Tris-HCl, pH 8.8 | ... | 5.0 | ml |
| 10% SDS | ... | 0.2 | ml |
| Acrylamide stock | ... | 8.0 | ml |

The above solution was mixed well and degassed for 3 minutes and then the following were added immediately.

| | | | |
|---|---|---|---|
| Freshly prepared ammonium persulphate | ... | 0.10 | ml |
| TEMED | ... | 0.01 | ml |

Mixed well and poured immediately between glass plates and a layer of water was added above the polymerising solution to quicken the polymerization process.

b) Preparation of stacking gel

| | | | |
|---|---|---|---|
| Double distilled water | ... | 6.1 | ml |
| Tris-HCl, pH 6.8 | ... | 2.5 | ml |
| 10% SDS | ... | 0.1 | ml |
| Acrylamide stock | ... | 1.3 | ml |

The solution was mixed well, degassed and the following were added.

| | | | |
|---|---|---|---|
| 10% ammonium persulphate | ... | 0.05 | ml |
| TEMED | ... | 0.01 | ml |

The water layered over the separating gel was removed and washed with a little electrode buffer and then the stacking gel was poured over the polymerized separating gel, after keeping the comb in position.

After polymerization the samples were loaded into the wells. The electrophoresis was performed at 40 V till the dye reached the separating gel. Then the voltage was increased to 140 V and continued till the dye reached the bottom of the gel. The gel was removed immediately after electrophoresis from the glass plates and incubated in the staining solution for overnight with uniform shaking. Then the gel was transferred to the destaining solution. The proteins appeared as bands and the gel was photograhed. The molecular weight of the polypeptides were calculated from a standard graph prepared for a 12 per cent gel using the standard markers (dalton marker, Sigma). The electrophoresis was performed in a Hoefer SE 600 series electrophoresis system.

#### 3.10.4.3 Electrophoretic analysis of leaf isozymes (Non-denaturing gel electrophoresis)

Electrophoresis of protein extracts from plant tissue, using different kinds of support media and buffer systems, allows separation of the multiple forms of enzymes (isozymes) on the basis of charge and/or molecular size. The resulting polymorphisms are useful as genetic markers. Isozyme analysis is a powerful technique for estimating genetic variability, identifying cultivars and germplasm accessions.

The present work was undertaken to study the enzyme alterations in primary leaves of cowpea and to elucidate the peroxidase isozyme profiles of 10 genotypes (4 susceptible and 6 resistant) selected during the course of screening for resistance from the germplasm accessions.

3.10.4.3.1 Enzyme extraction and assay

Soluble and ionically bound enzymes were extracted by grinding 5 g of tissue at 4°C in 10 ml of 0.1 M sodium phosphate buffer, pH 6.5 containing 1 mM phenylmethylsulphonylfluride (PMSF) with a mortar and pestle. The homogenates were incubated at 4°C for 1 h to complete the extraction, then filtered through 2 layers of cheese cloth and cleared by centrifugation at 20,000 g for 20 minutes. The resulting supernatant was assayed directly for peroxidase activity. Total protein was determined by the coomassie dye-binding assay, using Hitachi 200-20 spectrophotometer.

3.10.4.3.2 Isozyme separation and staining

Discontinuous anionic polyacrylamide gel electrophoresis was conducted under non-dissociating conditions as previously described by Wagih and Coutts, 1982a). Proteins extracted by phosphate buffer (pH 6.5) were separated by gel electrophoresis

on 7.5 per cent gel. The gel was prepared using the stock solution prepared for protein gel electrophoresis, with slight change in chemicals.

Reagents

A. Separating gel (7.5%)

| | | | |
|---|---|---|---|
| Acrylamide stock | ... | 7.50 | ml |
| Tris-HCl, pH 8.8 | ... | 7.50 | ml |
| Triton X 100 | ... | 0.20 | ml |
| Distilled water | ... | 14.53 | ml |

(Degassed well for 2-3 minutes)

| | | | |
|---|---|---|---|
| APS (10%) | ... | 0.10 | ml |
| TEMED | ... | 0.01 | ml |

B. Stacking gel (4%)

| | | | |
|---|---|---|---|
| Distilled water | ... | 6.1 | ml |
| Tris-HCl, pH 6.8 | ... | 2.5 | ml |
| Triton X 100 | ... | 0.1 | ml |
| Acrylamide stock | ... | 1.3 | ml |

(Degassed for 2-3 minutes)

| | | |
|---|---|---|
| APS (10%) | ... | 0.05 ml |
| TEMED | ... | 0.01 ml |

Electrode buffer and sample buffer were same as that of denaturing gel except that SDS was replaced by Triton X 100 (2%). Following electrophoresis, gels were incubated in the following solutions.

| | | | |
|---|---|---|---|
| Benzidine | ... | 2.08 | g |
| Acetic acid | ... | 18.00 | ml |
| Hydrogen peroxide (3%) | ... | 100.00 | ml |
| Water | ... | 80.00 | ml |

After the bands were stained sufficiently (bright blue colour), the reaction was arrested by immersing the gel into a large volume of 0.67 per cent sodium hydroxide or 7 per cent acetic acid solution for 10 minutes. The Rf values of each band were calculated.

3.10.5 Estimation of nucleic acids

The nucleic acid estimation was carried out based on the procedure described by Malik and Singh (1980). The leaf sample (200 mg) was homogenized in 1.5 ml of 10 per cent perchloric acid (PCA) in a glass homogenizer at 0°C and centrifuged at 10,000 rpm for 20 minutes. The supernatant was discarded and the residue was resuspended in 5 per cent cold PCA and centrifuged at 10,000 rpm. The supernatant was discarded and the residue was washed sequentially with 70 and 95 per cent ethanol and finally with boiling ethanol-ether (3:1) mixture twice and then with 0.2N cold PCA and centrifuged at 10,000 rpm immediately. The residue was resuspended in cold 2N PCA and stored at 4°C for 18 h. The suspension was then centrifuged at 10,000 rpm and the supernatant was collected. The residue was washed again with cold 2N PCA. After centrifugation, the supernatants were combined and the volume was made upto 5 ml with distilled water (Fraction I). This fraction contained ribonucleic acid (RNA) fraction and samples from this supernatant were used for quantitative estimation of RNA.

The residue was suspended in 1N PCA and heated at 70°C for 20 min and centrifuged. The supernatant was collected and retained. The residue was reextracted with hot 1N PCA, centrifuged and the supernatant was collected and pooled. The final

volume was made upto 5 ml (Fraction II) with distilled water and this fraction was used for estimation of Deoxyribonucleic acid (DNA).

The RNA and DNA in PCA extracts (Fraction I and II respectively) were estimated by measuring the absorbance at 260 nm in Hitachi 200-20 spectrophotometer.

The extinction coefficient of RNA (10,800 mol $l^{-1}cm^{-1}$) and DNA (8,780 mol $l^{-1}cm^{-1}$) was used to calculate the RNA and DNA content which were expressed in $\mu$mol $g^{-1}$ of leaf tissue on fresh weight basis.

### 3.10.6 Assay of enzymes

Application of antiviral principles (AVPs) and infection due to pathogens causes alterations in the biochemical activities in plants, especially in qualitative and quantitative alterations in enzyme activities. In the present investigation an attempt has been made to elucidate the changes in activities of different enzymes. The following treatments were given to both susceptible and resistant varieties.

1. Sprayed with *Bougainvillea spectabilis* leaf extract (BSLE)
2. Sprayed with BSLE + Inoculated with CAMV
3. Inoculated with CAMV
4. Uninoculated control

All treatments were given on 7 days old cowpea seedlings. Seedlings were inoculated artificially by mechanical inoculation 24 h after application of leaf extract (10%). The leaf samples were taken at 1, 5, 10 and 15 d after inoculation for the assay of peroxidase, polyphenol oxidase and phenylalanine ammonia-lyase and 2, 4, 6, 8 and 10 days for chitinase assay.

3.10.6.1 Peroxidase (PO)

One g of leaf sample was homogenized in 5 ml of 0.1M sodium phosphate buffer (pH 6.5) to which a pinch of polyvinyl pyrollidone (PVP) was added. The homogenization was done at 4°C using a mortar and pestle. The homogenate was filtered through muslin cloth and centrifuged at 10,000 rpm for 10 minutes at 4°C. The supernatant was used as the enzyme extract for the assay of PO activity.

Peroxidase activity was determined according to the procedure described by Srivastava (1987) with slight modifica-

tion. The reaction mixture consisting of 1.5 ml of 0.05M pyrogallol and 0.5 ml of enzyme extract was taken in both reference and sample cuvettes, mixed and kept in a spectrophotometer and the reading was adjusted to zero, at 420 nm. The enzyme reaction was started by adding 100 µl of 1 per cent hydrogen peroxide ($H_2O_2$) into the sample cuvette and the changes in absorbance of the reaction mixture was recorded at 30 sec intervals.

An unit of peroxidase activity was defined as the amount of enzyme which causes a change in optical density of 0.001 per minute (Langcake and Wickins, 1975). The peroxidase activity was expressed on a fresh weight basis as changes in absorbance per minute per g leaf tissue (Hammerschmidt *et al*., 1982).

3.10.6.2 *Polyphenol oxidase* (PPO)

Polyphenol oxidase activity was determined as per the procedure given by Mayer *et al*. (1965). The enzyme extract was prepared as per the procedure given for estimation of peroxidase.

The reaction mixture contained 1.5 ml of 0.1M sodium phosphate buffer (pH 6.5) and 0.5 ml of the enzyme extract (inactivated enzyme in reference cuvette) in the cuvettes and mixed well. The cuvettes were placed in a Hitachi 200-20

spectrophotometer and absorbance was set to zero. The reaction was started after adding 0.1 ml of 0.01M catechol into the sample cuvette. The changes in optical density was recorded at 30 sec intervals for 5 minutes. The PPO activity was expressed as changes in the OD of the reaction mixture per minute per g on fresh weight basis.

3.10.6.3 Phenylalanine ammonia-lyase (PAL)

Phenylalanine ammonia-lyase (PAL) activity was analysed based on the procedure described by Dickerson *et al*. (1984). The enzyme extract was prepared by homogenizing 1 g leaf sample in 5 ml of 0.1M sodium borate buffer, pH 8.8 containing a pinch of PVP, using chilled pestle and mortar. The homogenate was filtered through double layer cheese cloth and was centrifuged at 10,000 rpm for 20 minutes at 4°C. The supernatant was used for the assay of PAL activity. The reaction mixture contained 3.1 ml of 0.1M sodium borate buffer (pH 8.8), 0.2 ml of enzyme extract and 0.1 ml of 12 mM L-phenylalanine prepared in the same buffer. The blank was made by taking 3.1 ml of 0.1M sodium borate buffer (pH 8.8) and 0.2 ml of the enzyme extract. The reaction mixture and the blank were incubted at 40oC for 30 minutes and the reaction was stopped by adding 0.2 ml of 3N HCl. The absorbance was read at 290 nm in an Hitachi 200-20 spectrophotometer.

An extinction coefficient of 9630 $mole^{-1}cm^{-1}$ was determined for trans-cinnamic acid in 0.1M borate buffer, pH 8.8 (Zucker, 1965). The extinction coefficient was used to calculate the amount of product formed per minute. PAL activity was expressed in n moles of cinnamic acid produced per minute per g on fresh weight basis.

3.10.6.4 <u>Chitinase</u>

Chitinase activity was assayed colorimetrically following the method of Boller and Mauch (1988).

The colloidal chitin was prepared by the procedure described by Berger and Reynolds (1958). A sample (1 g) of chitin powder (passed through a 42 mesh sieve) was added slowly into 40 ml of concentrated hydrochloric acid at 4° C with vigorous stirring. After homogenous dispersion of chitin powder had been reached, the mixture was heated gently upto 37° C with moderate stirring. The viscosity of the mixture increased rapidly and then, within a few minutes, began to decrease and the appearance of the mixture became clearer. The mixture was then filtered through glasswool and the filtrate was poured into 400 ml of the deionized double distilled water at 4° C with stirring for 30 minutes. Within a few minutes the solution became turbid because of reprecipitation of chitin. Then the suspension was kept

ovenight below 5°C. The supernatant was then decanted out and the remaining mixture was filtered through Whatman No.2 filter paper. The residue was washed with water until the washings became neutral. The acid free residue was added in a few ml of deionized water and resuspended with vigorous stirring to prepare so called colloidal chitin solution. Chitin content of the solution was determined by drying a sample *in vacuo*. The final volume of the colloidal chitin solution was made upto give a concentration of 10 mg $ml^{-1}$ and sodium azide was added to the solution to a strength of 3 mM.

Snail gut enzyme was prepared as per the methods described by Cabib and Bowers (1971). Six hundred mg of the commercial lyophilized snail gut enzyme (Helicase, Sigma) was dissolved in 10 ml of 20 mM KCl and chromatographed on a Sephadex G-25 column (38 x 1.5 cm) using a 10 mM KCl solution containing 1 mM EDTA and adjusted to pH 6.8 for equilibration and elution. The first volume eluted after the void volume was collected and the final concentration was made upto 30 mg $ml^{-1}$.

The DMAB reagent was prepared by the procedure described by Reissig *et al*. (1955). One volume of stock solution of 8 g p-dimethylaminobenzaldehyde in 70 ml glacial acetic acid and 10 ml concentrated HCl was mixed with 9 volume of glacial acetic acid immediately before use.

Procedure

Leaf samples (300 mg) were homogenized in 1 ml of 10 mM sodium acetate buffer (pH 5.0) using pestle and mortar in an ice tray. The homogenate was centrifuged at 10,000 rpm for 20 min at 4°C and the supernatant was used as crude enzyme source for chitinase assay.

For the colorimetric assay of chitinase, 10 µl of 1M sodium acetate buffer (pH 4.0) and 0.5 ml of enzyme solution were pipetted into a 1.5 ml Eppendorf tube. The reaction was carried out at 37°C in a shaking water bath by the addition of 100 µl of colloidal chitin. The reaction of the mixture was stopped by centrifugation (10,000 g, 3 min) after 2 hours. An aliquot (0.3 ml) of the supernatant was pipetted into a glass reagent tube containing 30 µl of 1M potassium phosphate buffer (pH 7.1) and 20 µl of snail gut enzyme and was incubated for 1 h.

After incubation 70 µl of 1M sodium borate buffer (pH 9.8) was added to the reaction mixture and incubated in a boiling water bath for 3 min and then rapidly cooled in an ice water bath. After the addition of 2 ml of the DMAB reagent, the mixture was incubated for 20 minutes at 37°C. Immediately thereafter, optical density of the reaction mixture was measured at 585 nm using an Hitachi 200-20 spectrophotometer. The blank was

also maintained with the extraction buffer (sodium acetate buffer 10 mM, pH 5.0) instead of the enzyme extract. N-acetylglucoseamine (GlcNAc) was used as the standard (Boller *et al.*, 1983).

### 3.10.7 Estimation of phenolics

Leaf sample (200 mg) was extracted in 7.5 ml of warm 80 per cent ethanol. After cooling the extract was centrifuged at 10,000 rpm for 10 minutes. The volume of the supernatant was made upto 7.5 ml with 80 per cent ethanol (Lee and Tourneau, 1958) which was further used for estimation of total phenols and ortho-dihydroxy phenols (O.D.phenols).

#### 3.10.7.1 Total phenols

Total phenol content was estimated as per the procedure described by Bray and Thorpe (1954). To 1 ml aliquot of the ethanolic extract, 5 ml of distilled water and 250 µl of 1N Folin-Ciocalteau reagent were added. After incubating the mixture at 25°C for 3 minutes, 1 ml of saturated sodium carbonate ($Na_2CO_3$) solution was added and the mixture was further incubated for 15 minutes. The blue colour developed after incubation was read at 725 nm using Hitachi 200-20 spectrophotometer. A blank containing all the reagents except the extract was used to adjust

the absorbance to zero. The phenol content was expressed in catechol equivalents mg $g^{-1}$ of leaf tissue on fresh weight basis.

3.10.7.2 O.D. phenols

O.D. phenol content of the ethanolic extract was estimated by the method described by Johnson and Schaal (1952, 1957) employing Arnow's reagent. Arnow's reagent was prepared by dissolving 10 g of sodium nitrite ($NaNO_2$) and 10 g of sodium molybdate ($Na_4MoO_2$) in 100 ml of distilled water. The reagent was stored in a brown bottle (Arnow, 1937).

To 1 ml of the ethanolic extract, 1 ml each of 0.5N HCl and Arnow's reagent, 10 ml of distilled water and 2 ml of 1N NaOH was added. A reagent blank was maintained without the extract. soon after the addition of the alkali, a pinkish yellow colour develops. The absorbance of the solution was read at 515 nm using Hitachi 200-20 spectrophotometer. The O.D. phenol content was expressed in catechol equivalents mg $g^{-1}$ of leaf tissue on fresh weight basis.

3.11 MANAGEMENT OF CAMV

Management of CAMV was undertaken using virus inhibitory chemicals, plant products and insecticides. The

experiments were carried out in glasshouse and under field conditions. The field experiments were conducted at experimental farm of Agricultural College and Research Institute, Tamil Nadu Agricultural University, Coimbatore.

### 3.11.1 Management of CAMV using virus inhibitory chemicals

This experiment was done under glasshouse condition. Twenty five chemicals were screened for virus inhibition during the period of investigation. Healthy seedlings of *Chenopodium amaranticolor*, a local lesion host of CAMV, were transplanted in 12" clay pots and housed in a glasshouse. To detect the viral inhibitory activity of the chemicals, they were applied at different concentration with a paint brush to the upper surface of fully expanded leaves. Twenty four hours after application of the chemicals, the leaves of the test plants were dusted with 600 mesh carborundum powder and the virus inoculum was applied with the forefinger on the upper leaf surface.

All the experiments were performed in an insect-free glasshouse at about $22 \pm 6^{\circ}C$. The appearance of local lesion was monitored at weekly intervals and observations were taken as the number of lesions per $cm^2$. The data were analysed statistically by the test of comparison between the control and the individual treatment (check versus treatment) to test for the significance

of the chemicals (Snedecor, 1961). Per cent inhibition was calculated by the formula (C-T)/C x 100, where C is the number of lesions on control leaves and T is the number of lesions on treated leaves.

3.11.2 Management of CAMV disease using plant products

3.11.2.1 Screening of plant species for the presence of antiviral principles (AVPs)

Twenty five plant species were screened to detect the viral inhibitory activity against CAMV.

Preparation of AVPs

Fresh tissues of leaves were ground in a mortar and pestle with distilled water, at the rate of 10 ml/g plant tissue. The pulp was strained through two folds of cheese cloth and the filtrate was centrifuged at 3000 g for 15 minutes. The volume of the leaf extracts containing the AVPs were made upto a concentration of 10 per cent (w/v). This crude extract was used without further treatment.

Bioassy for inhibition of AVPS

To detect the viral inhibitory activity, the crude extracts were applied on leaves of test plants 24 h before inocu-

lation with CAMV. In another experiment, the leaf extracts were mixed with CAMV inoculum in equal amounts and the mixture was inoculated onto leaves of assay host. Leaves of control plants were similarly rubbed with distilled water before inoculation for the former experiment and virus inoculum mixed with equal volumes of distilled water for the latter. In preinoculation treatments before virus inoculation, leaves were washed with distilled water. The decrease in lesion number of virus titre in treated samples was compared with that of the virus in control.

All the experiments were performed in an insect-proof glasshouse at about 22 ± 6°C. Per cent inhibition was calculated.

#### 3.11.2.2 Ammonium sulphate fractionation of proteins from leaf extracts

Ammonium sulphate is particularly a useful salt for the fractional precipitation of proteins. Changes in the ammonium sulphate concentration of a solution can be brought about either by adding solid substance or by adding a solution of known saturation, generally, a fully saturated (100% solution).

The protein fractions were precipitated from aqueous extracts of *Mirabilis jalapa*, *Bougainvillea spectabilis*, *Ipomoea carnea* and *Prosopis chilensis*. The anti-plant viral protein fractions, active against mechanical transmission of CAMV were designated as MAP, BAP, IAP and PAP respectively.

Fresh tissues of leaves were ground in a mortar and pestle with distilled water at the rate of 10 ml/g fresh tissue. The homogenates were strained through two folds of cheese cloth and the filtrates were centrifuged at 5000 g for 15 minutes and the supernatants were collected. To the supernatants ammonium sulphate (solid) was added to adjust a saturation of 20, 40, 60, 80 and 100 per cent with stirring. The resulting precipitates were collected by centrifugation at 5000 g for 20 minutes and were dissolved in distilled water and made upto original volume. the protein content of the samples were analysed by coomassie dye-binding assay, using Hitachi 200-20 spectrophotometer.

The bioassay of each fraction was determined using the assay host *C.amaranticolor* and the range of inhibition was recorded.

### 3.11.2.3 Partial purification of BAP

*Bougainvillea* anti-plant viral protein (BAP) was purified partially by following the procedure described by Takanami *et al*. (1990) with slight modification.

The powder of leaf tissues was extracted with 20 volumes (v/w) of 10 mM phosphate buffer (PB), pH 7.2, containing

0.1 per cent 2-mercaptoethanol. The extract was centrifuged at 5,000 g for 15 minutes. The supernatant was collected and the residue was resuspended in 10 volumes of the extraction medium described above. The suspension was again centrifuged and the resulting supernatant was combined with that obtained from the first centrifugation. The supernatant was adjusted to 90 per cent saturation by addition of solid ammonium sulphate with stirring. The resulting precipitate was collected by centrifugation at 5,000 g for 20 minutes and was dissolved in 10 mM PB, pH 7.2, then dialyzed against the buffer. The precipitate formed during dialysis was removed by centrifugation at 10,000 g for 15 minutes, the dialyzate was frozen and lyophilized. The lyophilized material was used for further biochemical studies.

3.11.2.4 Effect of partially purified BAP in induction of defence mechanisms

The experimental material comprised of three cowpea varieties, *viz.*, CO 4 and C 152 - susceptible to CAMV and CO 6 - resistant to CAMV. The seedlings were raised in earthenware pots of 30 cm diameter in an insect-proof glasshouse. Treatments were given on 7-day old seedlings.

The lyophilized material of BAP (800 mg) was dissolved in 20 volumes (v/w) of 10 mM PB, pH 7.2, and was sprayed on 7-day

old seedlings of cowpea. Leaf samples were drawn at 1, 2, 3, 4 and 5 days after treatments for assessment of biochemical alteration in plants. The changes in activities of different enzymes *viz*., peroxidase, polyphenoloxidase and phenylalanine ammonia-lyase were assessed as described earlier.

### 3.11.3 Management of CAMV disease under field conditions

A field trial was laid out during December 1994 and the experimental details are furnished below.

| | |
|---|---|
| Design of experiment: | Randomized Block Design |
| Number of replications: | Three |
| Plot size: | 4 x 3 m |
| Cultivar: | CO 4 |

**Treatments**

$T_1$ *Bougainvillea spectabilis* leaf extract (BSLE) (10% aqueous solution)

$T_2$ *Mirabilis jalapa* leaf extract (MJLE) (10% aqueous solution)

$T_3$ *Ipomoea carnea* leaf extract (ICLE) (10% aqueous solution)

$T_4$ *Prosopis chilensis* leaf extract (PCLE) (10% aqueous solution)

$T_5$ Neem oil (3% aqueous solution)

$T_6$ Mahua oil (3% aqueous solution)

$T_7$ Monocrotophos (0.05% a.i., 1.5 ml/l)

$T_8$ Control (Water spray)

The spray volume under each treatment was at the rate of 500 l/per ha. Teepol (0.1% v/v) was added to spray solution under each treatment. Each treatment was applied three times at 15 days interval starting from 15 days after sowing.

Biometric observations *viz*., height of the plant, number of leaves, length of roots, and fresh and dry weights were taken on 30 and 60 days after sowing. The incidence of disease and aphid population was monitored 10, 30, 60 and 90 days after sowing. The yield parameters *viz*., number of grains per pod, hundred grain weight and total grain yield were taken during the field experiment.

### 3.11.4 Efficacy of plant extracts and insecticide on transmission of CAMV and survival of aphids

Pot culture experiments were undertaken in glasshouse condition to evaluate the efficacy of plant extracts *viz.*, BSLE, MJLE, ICLE and PCLE and an insecticide on the inhibition of CAMV disease and survival of insects. Plant extracts in the form of 10 per cent leaf extracts and 0.05 per cent monocrotophos were sprayed at the rate of 2.5 ml per plant on the primary leaves of 7-day old cowpea seedlings (CO 4). Twenty four hours after the application of extracts and insecticide, the plants were inoculated with groups of 5 viruliferous adult aphids on each cowpea seedling. Each treatment were replicated four times. Suitable controls were maintained by spraying distilled water. Mean incubation period, percentage of plants infected and survival of aphids in different treatments were determined.

## 3.12. SCREENING FOR SOURCES OF RESISTANCE TO CAMV IN COWPEA GERMPLASM

Two hundred and fifty five germplasm entries obtained from Pulses Breeding Station, Tamil Nadu Agricultural University, Coimbatore and 77 cowpea germplasm accessions from National Bureau of Plant Genetic Resources, and Regional Station, Vellanikkara were raised in earthenware pots in an insect-proof glasshouse. One week old plants were inoculated artificially by

mechanical inoculation. The per cent infection was recorded 14 days after inoculation and the plants were maintained for one month. For disease rating, the scale described by Ramiah (1978) was followed as mentioned below.

| Per cent infection | Reaction |
|---|---|
| Upto 10 | Resistant (R) |
| 11 - 30 | Tolerant (T) |
| 31 - 60 | Susceptible (S) |
| 61 - 100 | Highly susceptible HS) |

## 3.13. *IN VITRO* CALLUS CULTURE AND REGENERATION OF COWPEA

### 3.13.1 Explant preparation

Leaves and hypocotyl explants were used for the investigation. Primary leaves and hypocotyl regions of 7-day old, *in vitro* grown seeds of cultivars *viz*., CO 6, CO 4 and C 152 were used as explants. They were washed with sterile distilled water to remove the surface contaminants and were surface sterilized using 0.1 per cent (w/v) mercuric chloride for 3-4 minutes. The surface sterilized explants were again rinsed with sterile distilled water 3-4 times and were aseptically inoculated on culture media.

### 3.13.2 Callus induction medium

The basal medium chosen for this study was MS medium (Murashige and Skoog, 1962). Six levels (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg $l^{-1}$) of 2,4-D (auxin) were tried for callus induction in combination with a single level of (1.0 mg $l^{-1}$) of kinetin. Three per cent sucrose and 0.8 per cent agar were included in all the callus induction media composition.

### 3.13.3 Regeneration medium

The basal medium MS supplemented with five levels (1, 1.5, 2.0, 2.5 and 3.0 mg $l^{-1}$) of BAP and five levels (0, 0.5, 1.0, 1.5 and 2.0 mg $l^{-1}$) of NAA were used for regeneration of callus in the present study. Two per cent sucrose was included in all the callus regeneration media combination.

## 3.14. STATISTICAL ANALYSIS

The data obtained from the experiments conducted were statistically analysed in factorial completely randomized design, completely randomized design and randomized block design (Panse and Sukhatme, 1961).

# *EXPERIMENTAL RESULTS*

# CHAPTER 4

## EXPERIMENTAL RESULTS

### 4.1 SYMPTOMATOLOGY

The symptoms of CAMV on cowpea differ in different varieties and within the same variety. The variation was observed with the type of leaves, time of infection, climatic conditions and differences in individual plants.

On mechanical inoculation to 10-day old test plants, initial symptoms appeared on the second leaf, 14 days after inoculation. The first visible symptom was in the form of clearing of veins, which made their appearance at or near the base of the leaf and gradually spread over the entire leaf (Plates 1, 2, 3 and 7). In CO 4 and C 152 varieties, this vein-clearing was very much pronounced. After 10-14 days the early symptoms on the young inner leaves changed gradually into a conspicuous mottling. Typical mosaic mottling with dark green and light green patches was produced in all the subsequent leaves (Plate 4). The irregular light and dark green areas between the veins distorted the leaf surface. In some cases, the whole leaflets turned chlorotic and in others small chlorotic blotches were found. The irregular patches of light green contained islets of normal green, which were often parallel to the veins. The mosaic symptom was accompanied by slight convex cupping, arching or inward curling of the

margin of the leaflets. The affected leaves were often puckered and show deep marginal indentation. In CO 4 and C 152 varieties, infected plants exhibited dark green vein-banding, leaf distortion, blistering and stunting (Plates 5 and 6) and in Arka garima, Sharika and Pusa Dophasli, dark reddish brown discolouration was produced on the veins.

The symptoms were usually more severe in the upper portions of the plant. The affected leaflets were asymmetrical, reduced in size, wrinkled, crinkled, deformed, twisted and curled either inward or downward about the margin. In addition to the leaf symptoms, there was general stunting of the plants, shortening of the internodes and excessive branching. The pods were deformed and were common in V.unguiculata ssp. sesquipedalis (Plates 10 and 11).

The infected plants exhibited severe puckering, mottling and defoliation of the trifoliates and extensive necrotic streaks in the stem, sometimes leading to drying of the entire plant (Plates 8 and 9).

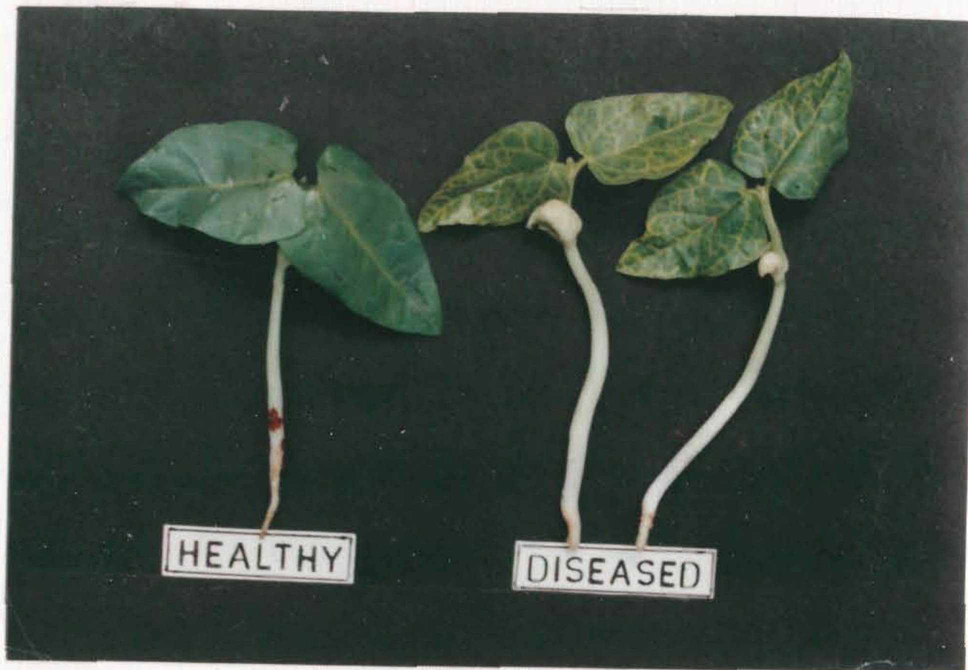



PLATE 1

PLATE 2

PLATE 3

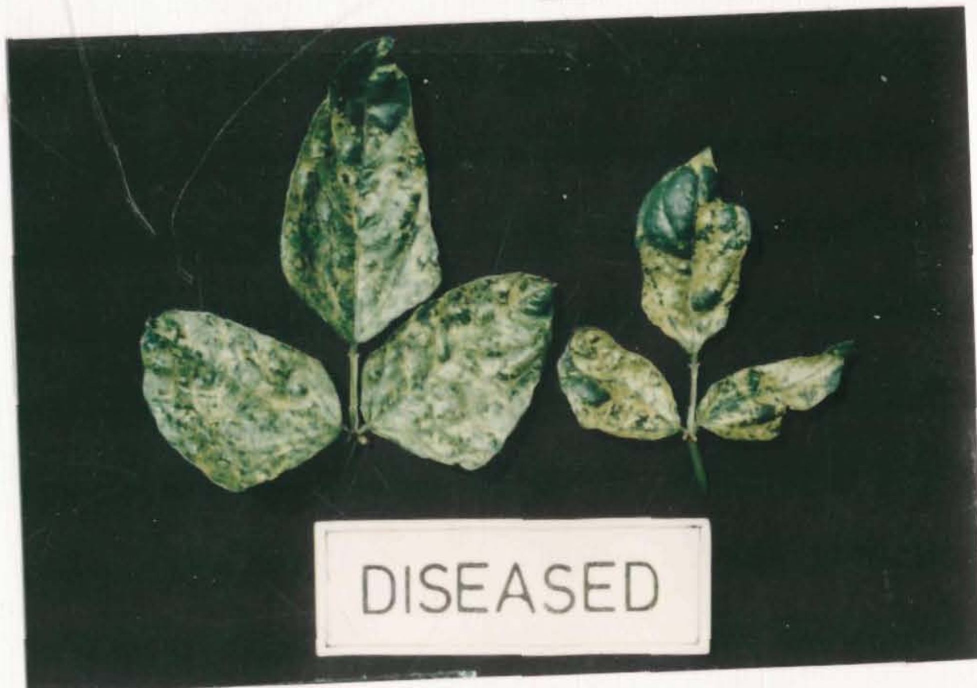



PLATE 4

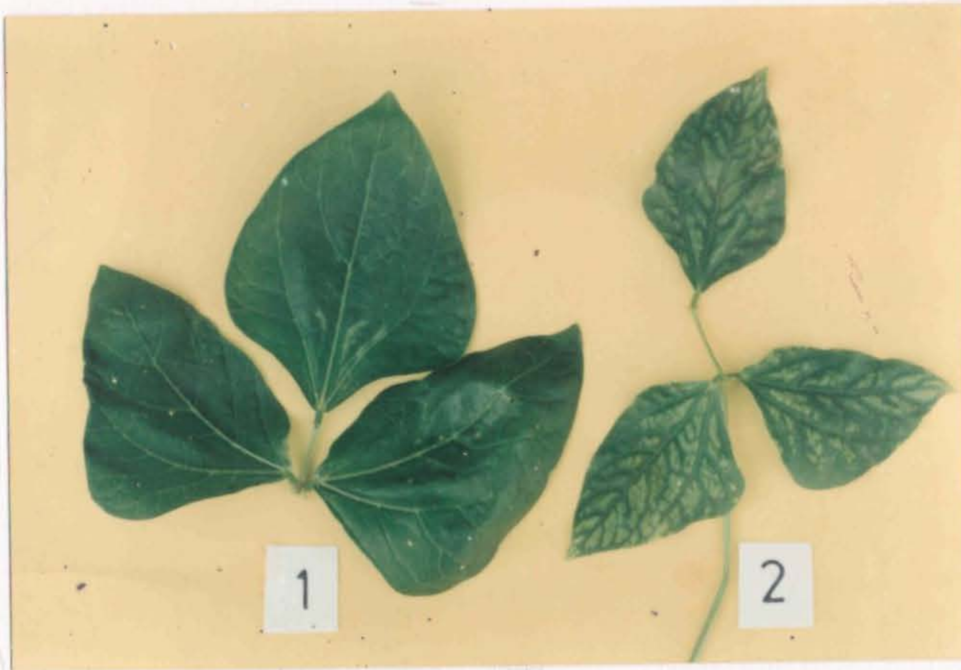



PLATE 5

PLATE 6

PLATE 7

. Healthy cowpea plant (Variety: Arka garima)

. Cowpea plant infected with CAMV showing mosaic mottling, puckering and deformed leaves. (Variety: Arka garima)

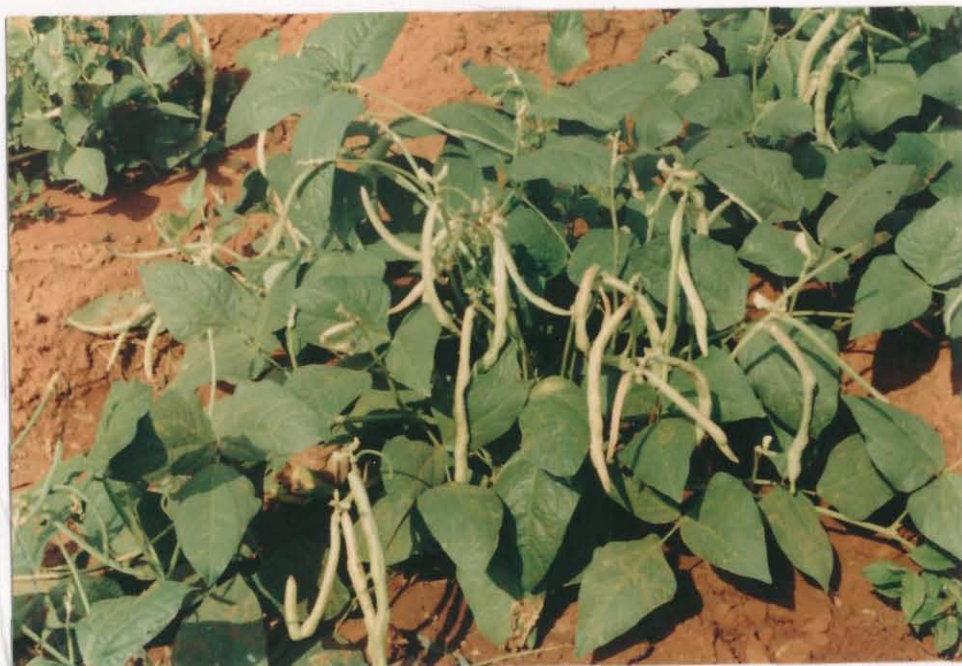


PLATE 8

PLATE 9

.0. Floral deformation of CAMV inoculated *Vigna unguiculata* ssp. *sesquipedalis*

11. Excessive branching of internodes and floral deformation of CAMV inoculated *V. unguiculata* ssp. *sesquipedalis* [2], [1] healthy pods.

PLATE 10

PLATE 11

## 4.2 TRANSMISSION

### 4.2.1 Mechanical transmission

The virus was found to be transmitted successfully through mechanical inoculation. Typical symptoms appeared 12-14 days after inoculation. Percentage of transmission varied with the extraction media used (Table 1). The potassium phosphate buffer 0.1M, pH 7.5 was associated with maximum infection (290.33% increase over control), followed by 0.1M sodium phosphate buffer, pH 7.4 (198.55% increase over control).

#### 4.2.1.1 Effect of different buffers on infectivity and stability of CAMV

With a view to evaluate the efficiency of different buffers such as citrate phosphate buffer pH 7.0, sodium phosphate buffer pH 7.0 and 7.4, potassium phosphate buffer pH 7.5, tris-HCl buffer pH 7.2, borate buffer pH 7.6 and cacodylate buffer pH 7.0 were tested at 0.1M concentrations as extracting fluids. The standard extracts of the virus prepared in these buffers as well as in distilled water were inoculated after 4, 8, 12 and 24 h on young seedlings of cowpea. The results are presented in Table 2.

It was observed that CAMV was infective in all buffers and in water extract for 8 h. Sodium phosphate buffers pH 7.0

Table 1. Effect of different buffers on infectivity of CAMV on *Chenopodium amaranticolor*

| Sl. No. | Buffer | Molar concen-tration | pH | Mean No. of lesions | Per cent increase/ decrease over control |
|---|---|---|---|---|---|
| 1. | Citrate phosphate | 0.1 | 7.0 | 90.2 | 117.87 |
| 2. | Sodium phosphate | 0.1 | 7.0 | 108.8 | 162.80 |
| 3. | Sodium phosphate | 0.1 | 7.4 | 123.6 | 198.55 |
| 4. | Potassium phosphate | 0.1 | 7.5 | 161.6 | 290.33 |
| 5. | Tris-HCl buffer | 0.1 | 7.2 | 35.2 | - 14.98 |
| 6. | Borate buffer | 0.1 | 7.6 | 45.4 | 9.66 |
| 7. | Cacodylate buffer | 0.1 | 7.0 | 93.4 | 125.60 |
| 8. | Control (Distilled water) | | - | 41.4 | 0.00 |
| | C.D. (P = 0.05) | | | 14.537 | |

Table 2. Effect of different buffers on infectivity and stability of CAMV on cowpea

| Sl. No. | Buffer | pH | Per cent infection after different periods of storage* (27-32°C) | | | |
|---|---|---|---|---|---|---|
| | | | 4h | 8h | 12h | 24h |
| 1. | Citrate phosphate | 7.0 | 50 | 10 | 0 | 0 |
| 2. | Sodium phosphate | 7.0 | 100 | 60 | 30 | 10 |
| 3. | Sodium phosphate | 7.4 | 100 | 60 | 30 | 10 |
| 4. | Potassium phosphate | 7.5 | 100 | 90 | 80 | 30 |
| 5. | Tris-HCl buffer | 7.2 | 40 | 10 | 0 | 0 |
| 6. | Borate buffer | 7.6 | 50 | 10 | 0 | 0 |
| 7. | Cacodylate buffer | 7.0 | 60 | 10 | 0 | 0 |
| 8. | Control | - | 70 | 10 | 0 | 0 |

* Based on 10 plants inoculated

and 7.4 and potassium phosphate buffer pH 7.5 were found to be superior to other treatments tried. Extraction with potassium phosphate buffer pH 7.5 and sodium phosphate buffers pH 7.0 and 7.4 retained the infectivity even after 24 h although the infectivity was reduced to only 30, 10 and 10 per cent respectively.

### 4.2.2 Seed transmission

The results on the transmission of CAMV through seeds of cowpea showed that the virus was also transmitted through seeds in all 10 cowpea cultivars tested (Table 3). The extent of seed transmission ranged from 5.77 to 15.38 per cent. A maximum seed transmission (15.38%) was observed in cv. Pusa Dophasli and it was minimum (5.77%) was in cv. Pusa Komal.

### 4.2.3 Insect transmission

Studies on insect transmission of CAMV was carried out using five vectors *viz*., *A.craccivora*, *A.gossypii*, *M.persicae*, *B.tabaci* and *C.trifurcata*, to find out their ability to transmit the virus. The susceptible variety CO 4 was used for the investigation. The results indicated that CAMV was more readily transmitted by three aphid species. The observations showed that the highest per cent transmission (93.33) was obtained with *A.craccivora* among the three aphid species tested. *M.persicae*

Table 3. Seed transmission of CAMV in susceptible varieties

| Sl. No. | Varieties | No. of seeds sown | No. of seeds germinated | No.of plants infected | Per cent trans-mission |
|---|---|---|---|---|---|
| 1. | CO 4 | 300 | 270 | 33 | 12.22 |
| 2. | C 152 | 450 | 380 | 26 | 6.84 |
| 3. | Arka garima | 80 | 72 | 8 | 11.11 |
| 4. | Malika | 42 | 36 | 3 | 8.33 |
| 5. | Sharika | 126 | 108 | 10 | 9.26 |
| 6. | Kanakamony | 208 | 170 | 12 | 7.06 |
| 7. | Pusakomal | 56 | 52 | 3 | 5.77 |
| 8. | Pusa Dophasli | 28 | 26 | 4 | 15.38 |
| 9. | Sel.263 (Ludhiana) | 20 | 16 | 2 | 12.50 |
| 10. | Sel.2-1 (Faizabad) | 40 | 32 | 2 | 6.25 |

and A.gossypii gave 30.0 and 26.67 per cent transmission respectively. However B.tabaci and C.trifurcata failed to transmit the virus (Table 4).

## 4.3 VIRUS-VECTOR RELATIONSHIP

The investigations on the virus-vector relationship of CAMV disease was undertaken to standardize the acquisition access period, inoculation access period, preacquisition starvation period, preinoculation starvation period, number of aphids required for transmission and persistence of the virus in the vector. The results are as follows.

### 4.3.1 Effect of acquisition access period

The results showed that a short acquisition access period of 5 minutes was sufficient for the aphids to become viruliferous. The optimum acquisition feeding period which gave the maximum per cent infection, viz., 90 per cent was found to be 30 minutes (Table 5).

### 4.3.2 Effect of inoculation access period

The data indicated that the viruliferous aphids were capable of transmitting the virus even with one minute inocula-

Table 4. Insect transmission of CAMV

| Sl. No. | Vector | Plants infected/ plants exposed (No.) | Plants infected (%) |
|---|---|---|---|
| 1. | *Aphis craccivora* | 28/30 | 93.33 |
| 2. | *A.gossypii* | 8/30 | 26.67 |
| 3. | *Myzus persicae* | 9/30 | 30.00 |
| 4. | *Bemisia tabaci* | 0/30 | 0 |
| 5. | *Cerotoma trifurcata* | 0/30 | 0 |

Table 5. Effect of acquisition access period on the transmission of CAMV by *Aphis craccivora*

| Acquisition access period | Plants infected/ plants exposed* (No.) | Plants infected (%) |
|---|---|---|
| 1 min | 0/20 | 0 |
| 5 min | 1/20 | 5 |
| 10 min | 5/20 | 25 |
| 20 min | 11/20 | 55 |
| 30 min | 18/20 | 90 |
| 1 h | 18/20 | 90 |
| 3 h | 17/20 | 85 |
| 6 h | 16/20 | 80 |
| 24 h | 17/20 | 85 |

* Groups of five adults were transferred to each cowpea seedlings and allowed a 24 h inoculation access period

tion feeding on the test plant. Maximum transmission of 80 per cent was obtained with 30 minutes inoculation access period (Table 6).

### 4.3.3 Effect of preacquisition starvation period

The preliminary fasting of aphids resulted in an increase in per cent infection. The vector could transmit the virus even without starvation but the starvation enhanced the per cent infection. Maximum infection of 88 per cent was obtained with one hour fasting and there was a gradual decline in per cent infection as the starvation period was increased (Table 7).

### 4.3.4 Effect of pre inoculation starvation

It was observed that starvation of 30 minutes or more after acquisition decreased the per cent infection. A maximum infection of 90 per cent was obtained when the aphids were immediately transferred after acquisition access period and the least percentage of infection was obtained when the aphids were allowed a preinoculation starvation of 5 h (Table 8).

Table 6. Effect of inoculation access period on the transmission of CAMV by *A.craccivora*

| Inoculation access period# | Plants infected/ plants exposed* (No.) | Plants infected (%) |
|---|---|---|
| 1 min | 1/25 | 4 |
| 5 min | 10/25 | 40 |
| 10 min | 12/25 | 48 |
| 15 min | 18/25 | 72 |
| 30 min | 20/25 | 80 |
| 1 h | 16/25 | 64 |
| 6 h | 12/25 | 48 |
| 12 h | 15/25 | 60 |
| 24 h | 10/25 | 40 |

# A 30-min acquisition access period was given to all insects

* Groups of five insects were released on each cowpea seedling

Table 7 . Effect of a preacquisition starvation period on the transmission of CAMV by *A.craccivora*

| Pre-acquisition starvation period# | Plants infected/ plants exposed* (No.) | Plants infected (%) |
|---|---|---|
| None | 15/25 | 60 |
| 30 min | 18/25 | 72 |
| 1 h | 22/25 | 88 |
| 2 h | 16/25 | 64 |
| 3 h | 12/25 | 48 |
| 4 h | 14/25 | 56 |
| 5 h | 10/25 | 40 |
| 6 h | 12/25 | 48 |

# All insects were given a 30-min acquisition access period after starvation and a 2-h inoculation access period

* Five adults were transferred to each test plant

Table 8. Effect of a preinoculation starvation period on the transmission of CAMV by *A. craccivora*

| Preinoculation starvation period* | Plants infected/ plants exposed (No.) | Plants infected (%) |
|---|---|---|
| None | 18/20 | 90 |
| 30 min | 16/20 | 80 |
| 1 h | 10/20 | 50 |
| 2 h | 10/20 | 50 |
| 3 h | 6/20 | 30 |
| 4 h | 2/20 | 10 |
| 5 h | 1/20 | 5 |
| 6 h | 0/20 | 0 |

* All insects were given 1 h pre-acquisition starvation, 30 min acquisition access period and a 2-h inoculation access period

4.3.5 Number of aphids required for transmission

It is seen from the results that even when a single adult apterous viruliferous aphid was used, the transmission of CAMV was to an extent of 5 per cent. The optimum number of aphids required to produce maximum infection (80 per cent) was found to be five (Table 9).

4.3.6 Persistence of CAMV in *A.craccivora*

The data in Table 10 indicated that successful infection could be obtained up to the fourth plant of the first series in which the aphids were transferred at intervals of 30 minutes and up to the third plant, when the interval was increased to 1 h. When the interval was increased to 1 h 30 minutes the infection was obtained up to the third plant and up to the second plant of the series when the interval was increased to 2 h. In all the other cases only the first plant of the series got infection, indicating that in most cases the viruliferous nature of the vector was lost after 2 h.

Table 9. Number of aphids required for transmission

| No. of aphids per plant* | Plants infected/ plants exposed (No.) | Plants infected (%) |
|---|---|---|
| 1 | 1/20 | 5 |
| 3 | 8/20 | 40 |
| 5 | 16/20 | 80 |
| 10 | 16/20 | 80 |
| 15 | 12/20 | 60 |
| 20 | 14/20 | 70 |

* All insects were given 1 h pre-acquisition starvation and a 30-min acquisition access period and a 2-h inoculation access period

Table 10. Persistence of CAMV in *A.craccivora*

| Feeding period on each test plant | | Infection produced in successive transfers | | | | |
|---|---|---|---|---|---|---|
| | | Serial number of plants inoculated | | | | |
| | | 1 | 2 | 3 | 4 | 5 |
| 30 min | a | + | + | + | + | - |
| | b | + | + | + | - | - |
| | c | + | + | + | + | - |
| 1 h | a | + | + | + | - | - |
| | b | + | + | + | - | - |
| | c | + | + | - | - | - |
| 1 h 30 min | a | + | + | + | - | - |
| | b | + | - | - | - | - |
| | c | + | + | + | - | - |
| 2 h | a | + | + | - | - | - |
| | b | + | - | - | - | - |
| | c | + | - | - | - | - |
| 2 h 30 min | a | + | - | - | - | - |
| | b | + | - | - | - | - |
| | c | - | - | - | - | - |
| 3 h | a | - | - | - | - | - |
| | b | + | - | - | - | - |
| | c | - | - | - | - | - |

## 4.4 HOST RANGE OF THE VIRUS

The results on host range and reactions of different hosts to CAMV are shown in Table 11.

The CAMV was inoculated by mechanical inoculation on 48 plant species belonging to 11 families. Minimum of five seedlings were inoculated in each case. The plants which did not show symptoms after four weeks were indexed on *C.amaranticolor* by back inoculation to determine if they were symptomless carriers of the virus. The virus was, however, found to have its host range restricted to four families *viz*., Amaranthaceae, Chenopodiaceae, Labiatae and Leguminosae. The virus produced systemic mosaic symptoms on *Canavalia ensiformis*, *Cyamopsis tetragonoloba*, *Glycine max*, *Phaseolus lunatus*, *P.trilobus*, *P.vulgaris*, *Pisum sativum* and *Psophocarpus tetragonalobus* and chlorotic local lesions on *C.amaranticolor*, *C.album*, *C.quinoa*, *Gomphrena globosa* and *Ocimum basilicum* (Plates 12 to 25).

## 4.5 ANATOMICAL STUDIES

The results of histopathological studies of healthy and virus infected apical meristems and leaves are depicted in Plates 26a to 26f.

Table 11. Host range of CAMV

| Sl. | Plant species | Plants infected/ plants inoculated | Per cent infection | Incubation period (days) | Symptoms | Family |
|---|---|---|---|---|---|---|
| 1. | Acalypha indica L. | 0/10 | - | - | NS | Euphorbiaceae |
| 2. | Amaranthus caudatus L. | 0/10 | - | - | NS | Amaranthaceae |
| 3. | A.gangeticus L. | 0/10 | - | - | NS | " |
| 4. | A.spinosus L. | 0/10 | - | - | NS | " |
| 5. | Arachis hypogea L. | 0/10 | - | - | NS | Leguminosae |
| 6. | Beta vulgaris L. | 0/10 | - | - | NS | Chenopodiaceae |
| 7. | Cajanus cajan (L.) Millsp. | 0/30 | - | - | NS | Leguminosae |
| 8. | Canavalia ensiformis DC. | 10/10 | 100 | 12-18 | M,Mt. | " |
| 9. | Chenopodium album L. | 10/10 | 100 | 3-5 | ClL | Chenopodiaceae |
| 10. | C.amaranticolor Coste & Reyn. | 10/10 | 100 | 3-5 | ClL | " |
| 11. | C.ambrosoides L. | 0/10 | - | - | NS | " |
| 12. | C.murale L. | 0/10 | - | - | NS | " |
| 13. | C.quinoa Willd. | 10/10 | 100 | 3-5 | ClL | " |
| 14. | Cucumis melo L. | 0/10 | - | - | NS | Cucurbitaceae |
| 15. | Cyamopsis tetragonaloba (L.) Taub. | 10/10 | 100 | 12-18 | M,Mt. | Leguminosae |
| 16. | Datura metel L. | 0/10 | - | - | NS | Solanaceae |
| 17. | D.stramonium L. | 0/10 | - | - | NS | " |
| 18. | Desmodium tortuosum Dest. | 0/10 | - | - | NS | Leguminosae |
| 19. | Dolichos biflorus L. | 0/30 | - | - | NS | " |
| 20. | Dolichos lablab L. | 0/30 | - | - | NS | " |
| 21. | Flavaria australasica Hook. | 0/ 5 | - | - | NS | Compositae |
| 22. | Glycine max (L.) Merr. | 10/10 | 100 | 12-15 | M,Mt. | Leguminosae |
| 23. | Gomphrena decumbens Jacq. | 0/10 | - | - | NS | Amaranthaceae |

| Sl. | Plant species | Plants infected/ plants inoculated | Per cent infection | Incubation period (days) | Symptoms | Family |
|---|---|---|---|---|---|---|
| 24. | *G.globosa* L. | 10/10 | 100 | 20-22 | CIL | " |
| 25. | *Lactuca runcinata* DC. | 0/ 5 | - | - | NS | Compositae |
| 26. | *Macroptelium atropurpureum* L. | 0/10 | - | - | NS | Leguminosae |
| 27. | *Nicotiana tabacum* L. | 0/30 | - | - | NS | Solanaceae |
| 28. | *N.glutinosa* L. | 0/30 | - | - | NS | " |
| 29. | *N.benthamiana* Domain. | 0/30 | - | - | NS | " |
| 30. | *N.clevelandii* Gray. | 0/30 | - | - | NS | " |
| 31. | *Ocimum basilicum* L. | 10/10 | 100 | 20-24 | ClL | Labiatae |
| 32. | *O.sanctum* L. | 0/10 | - | - | NS | " |
| 33. | *Parthenium hysterophorus* L. | 0/20 | - | - | NS | Asteraceae |
| 34. | *Phaseolus lunatus* L. | 10/10 | 100 | 10-18 | M,Mt. | Leguminosae |
| 35. | *P.trilobus* L. | 10/10 | 100 | 10-18 | M,Mt. | " |

| Sl. | Plant species | Plants infected/ plants inoculated | Per cent infection | Incubation period (days) | Symptoms | Family |
|---|---|---|---|---|---|---|
| 36. | *P.vulgaris* L. | 10/10 | 100 | 10-18 | M,Mt. | " |
| 37. | *Physalis minima* L. | 0/10 | - | - | NS | Solanaceae |
| 38. | *Pisum sativum* L. | 5/15 | 33.33 | 10-18 | M | Leguminosae |
| 39. | *Psophocarpus tetragonalobus* L. | 10/10 | 100 | 10-18 | M | " |
| 40. | *Sesamum indicum* L. | 0/10 | - | - | NS | Pedaliaceae |
| 41. | *Solanum melongena* L. | 0/10 | - | - | NS | Solanaceae |
| 42. | *S.nigrum* L. | 0/25 | - | - | NS | Solanaceae |
| 43. | *Trianthema portulacastrum* L. | 0/10 | - | - | NS | Aizoaceae |
| 44. | *Tridax procumbens* L. | 0/20 | - | - | NS | Compositae |
| 45. | *Vernonia cinerea* Less. | 0/10 | - | - | NS | " |
| 46. | *Vigna mungo* (L.) Hopper. | 0/20 | - | - | NS | Leguminosae |
| 47. | *V.radiata* (L.) Wilczek. | 0/20 | - | - | NS | " |
| 48. | *Zinnia elegans* Jacq. | 0/10 | - | - | NS | Compositae |

ClL = Chlorotic local lesion
M = Mosaic
Mt = Mottling
NS = No symptoms

PLATE 12

: 14. Chlorotic local lesions on *C. quinoa*

: 15. Necrotic local lesions on *Gomphrena globosa*.

PLATE 14

PLATE 15

PLATE 16

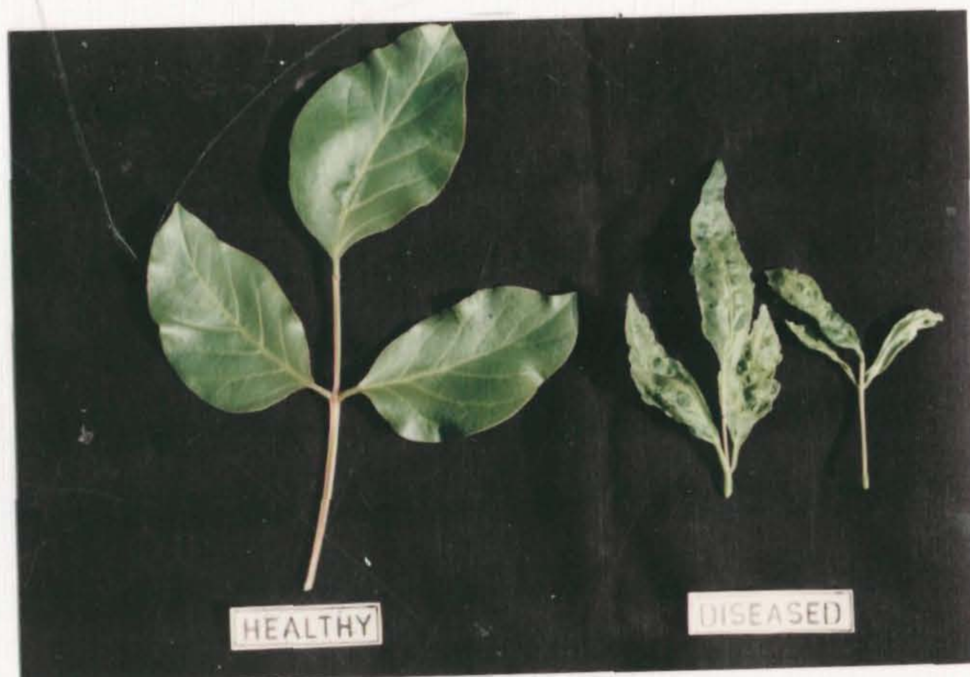



PLATE 17

ATE 18. Mosaic mottling and distortion of leaves of CAMV inoculated *Phaseolus vulgaris*.

ATE 19. Mosaic mottling and leaf distortion of CAMV inoculated *Phaseolus lunatus*.

PLATE 18

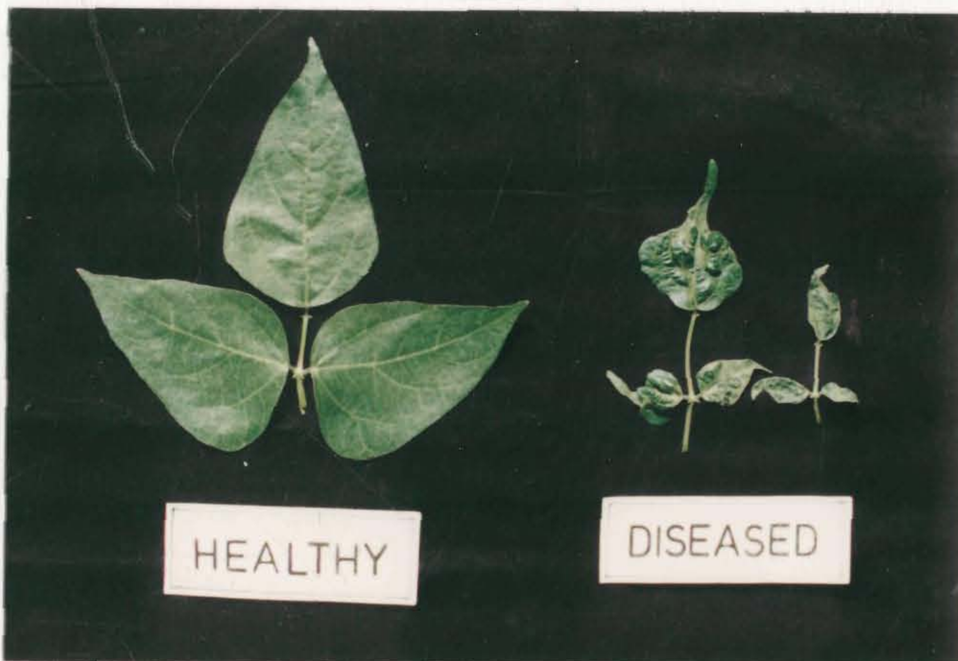



PLATE 19

.ATE 20. Mosaic symptoms with stunting in *Pisum sativum* inoculated with CAMV.

ATE 21. CAMV inoculated *Cyamopsis tetragonaloba* showing curling and puckering of leaves.

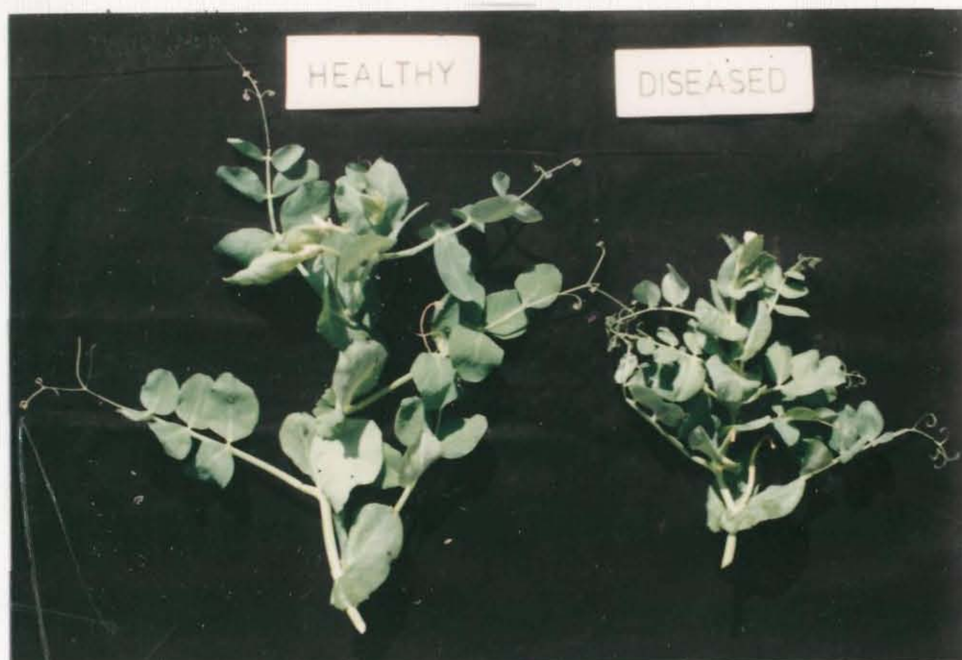



PLATE 20

PLATE 21

PLATE 22

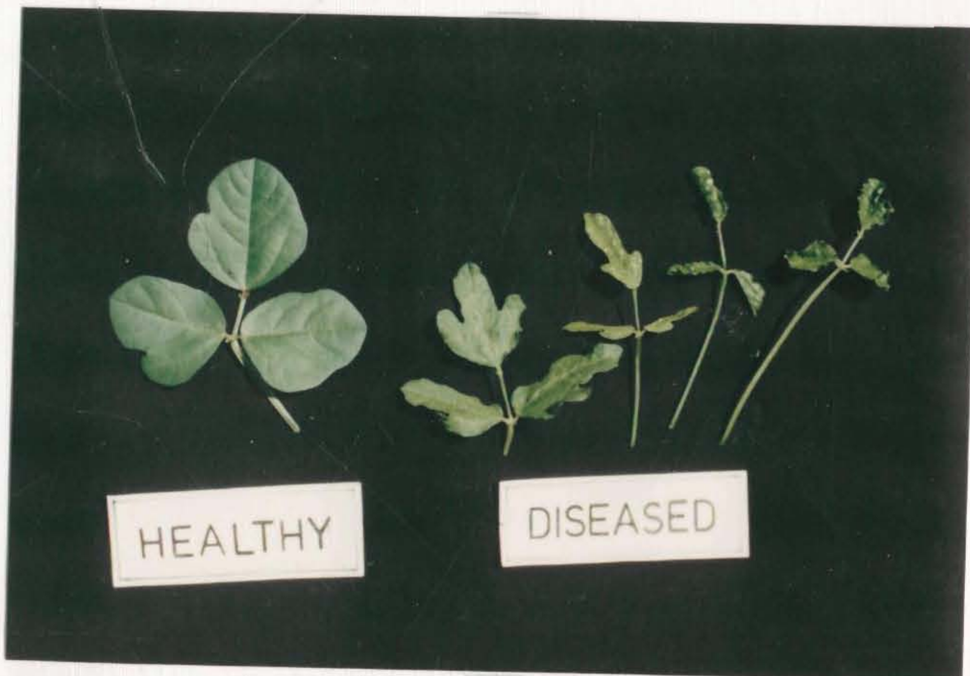



PLATE 23

PLATE 24. Mosaic symptom in Glycine max inoculated with CAMV.

PLATE 25. Leaf distortion in CAMV inoculated Psophocarpus tetragonalobus.

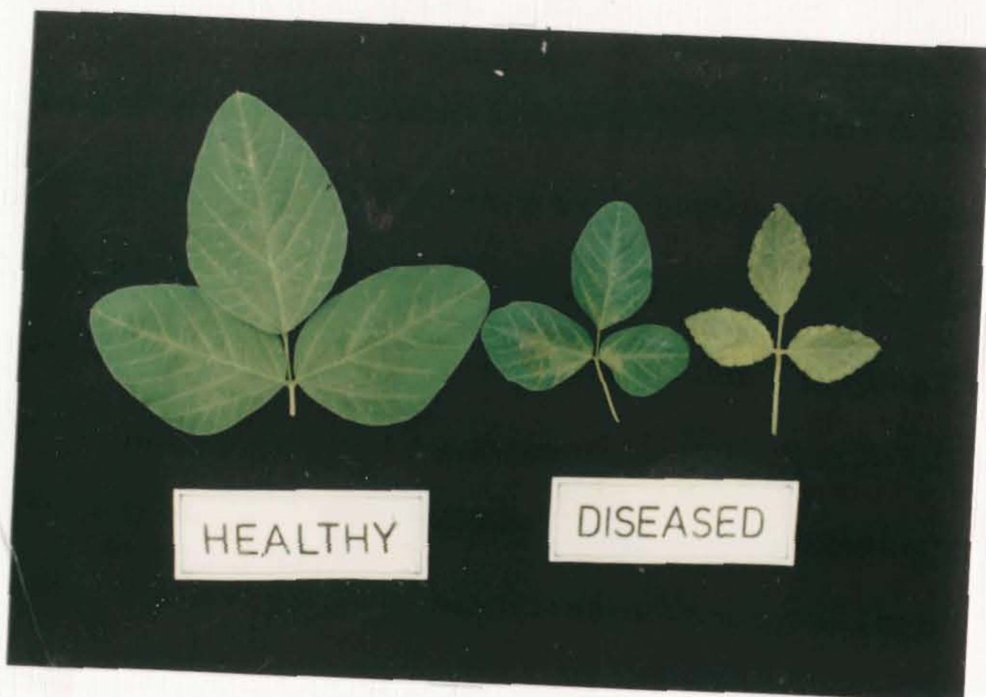



PLATE 24

PLATE 25

PLATE 26. HISTOPATHOLOGY OF CAMV INFECTED TISSUES

26a. Longitudinal section (LS) of healthy apical meristem stained with haematoxylin.

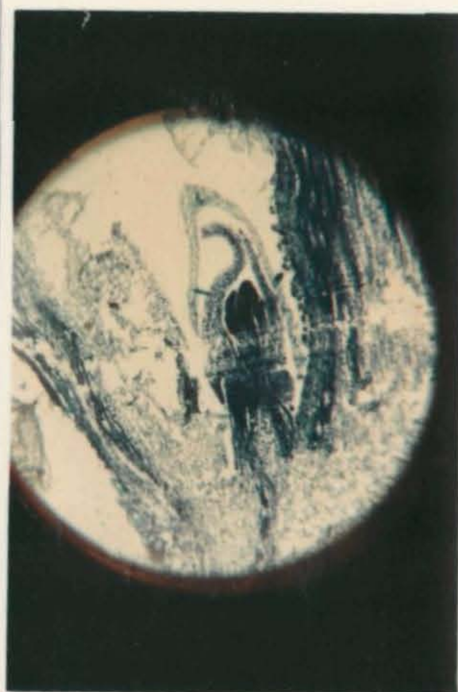
26b. LS of diseased apical meristem stained with haematoxylin.

26c. } LS of diseased apical meristem stained
26d. } with Azure A.

PLATE 26

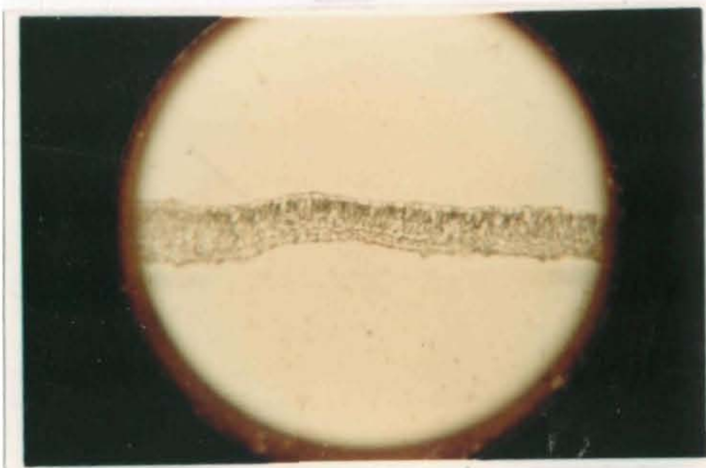
26a

26b

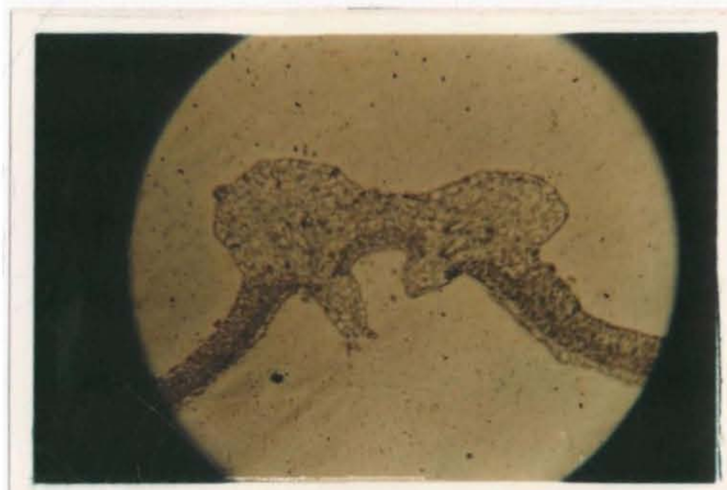


26e. Cross section (CS) of a healthy leaf stained with haematoxylin.

26f. CS of a diseased leaf showing proliferation of infected tissue, stained with haematoxylin.



26 e



26 f

The longitudinal sections (LS) of apical meristems of healthy and diseased cowpea plants clearly indicated that the growth was retarded in virus infected plants. There was severe curling of leaf primordia in diseased meristem tip and the growing point was poorly developed, when compared to healthy.

The infected leaf tissues also showed severe distortion of cells. There was clear difference in the cross sections (CS) of healthy and diseased leaves. There was heavy proliferation of cells in the infected tissues as evident from the Plates.

## 4.6 DETECTION OF CAMV

### 4.6.1 Detection of CAMV by DAC-ELISA

In order to find out the identity of the virus infecting cowpea grown in Coimbatore, the infected plants were tested by employing DAC-ELISA. This experiment was performed using polyclonal antisera produced against CAMV and monoclonal antibodies raised against CAMV and BICMV, obtained from IITA, Nigeria.

For serological identification, crude extracts from infected plants were used as mentioned in Materials and Methods.

The results of the experiment (Table 12) revealed that the CAMV MAbs, 5H5 and 7D9 showed a high reactivity towards the virus isolate from Coimbatore. The absorbance at 405 nm ($A_{405}$) was 0.861 and 0.974 respectively. The remaining two MAbs 1F5 and 6C10 homogeneously recognised the isolate and its $A_{405}$ values were 0.720 and 0.730 respectively.

The isolate was also specifically detected by the BICMV MAbs, viz., 15E6, 12E9, 10G5 and 16G5 and their absorbance at 405 nm was 0.346, 0.318, 0.286 and 0.292 respectively (Fig 1).

The wells which received the CAMV AbR showed a high reactivity towards the virus isolate and they read an average absorbance of 1.115 at $A_{405}$ nm. The healthy controls were associated with an average absorbance of 0.118 and the buffer control read 0.048 at $A_{405}$ nm (Plate 27).

4.6.2 Detection of CAMV by NCM-ELISA

The CAMV was detected in sap extracts of infected cowpea leaves and body fluid of viruliferous *A.craccivora* using a modified indirect enzyme-linked immunosorbent assay on nitrocellulose membrane (NCM-ELISA) described by Lizarraga and Fernandez-Northcote (1989). Antigen dilutions of 1:10, 1:50, 1:100 and 1:1000 were used for detecting the virus in CAMV infected leaves

Table 12. Reaction of CAMV isolate in enzyme-linked immunosorbent assay (ELISA) measured by using monoclonal and polyclonal antisera to CAMV and BICMV

| Treatments | | | Absorbance |
|---|---|---|---|
| I. Monoclonal antibodies | | | |
| i) | 5H5 | CAMV-Fenkan | 0.861 |
| ii) | 1F5 | CAMV-Monguno | 0.720 |
| iii) | 6C10 | CAMV-70-12 | 0.730 |
| iv) | 7D9 | CAMV-Nkeji | 0.974 |
| v) | 15E6 | BICMV-81.1 | 0.346 |
| vi) | 12F9 | BICMV | 0.318 |
| vii) | 10G5 | BICMV-81.11 | 0.286 |
| viii) | 16G5 | BICMV-81.11 | 0.292 |
| II. Polyclonal antibody | | | |
| i) | CAMV | | 1.115 |
| III. Control | | | |
| i) | Healthy control | | 0.118 |
| ii) | Buffer control | | 0.048 |

Fig.1 Reaction of CAMV isolate in enzyme-linked immunosorbent assay(ELISA)

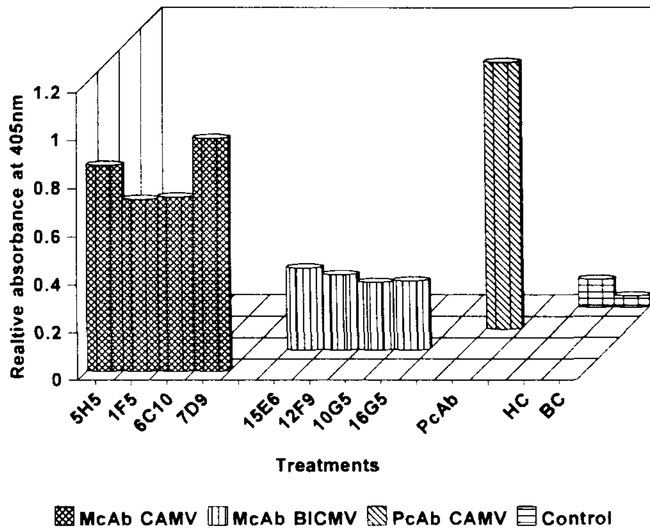


PLATE 27. Detection of CAMV in infected cowpea leaves by DAC-ELISA.

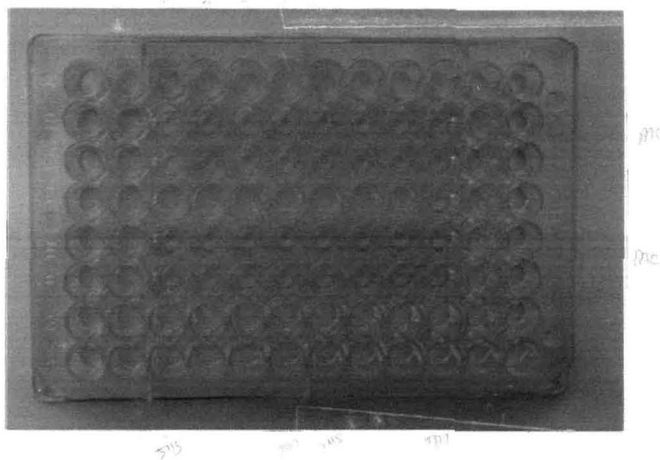


PLATE 27

and for detecting the virus in viruliferous aphid vector, extracts obtained by crushing aphids at the rate of 1, 5, 10 and 20 insects per 10 µl of antigen extraction buffer were used. Ten µl drops of antigen extracts were used for spotting onto the nitrocellulose membrane. Polyclonal antibodies for CAMV produced in rabbits and alkaline phosphatase conjugated goat antirabbit immunoglobulins were used for detecting the antigen.

The results of NCM-ELISA indicated that the CAMV could be detected in leaf extracts of infected plants and in viruliferous aphids. The positive reactions appeared as bluish purple spots and the negative reactions were colourless. There was colour development even with the highest dilutions (1:1000) of sap from infected cowpea leaves (Plate 28) and with body fluid of viruliferous aphids (Plate 29). The intensity of the colour decreased as the dilution was increased. There was no colour development when the extracts from healthy plants and non-viruliferous aphids were used as antigen source.

4.6.3 Electron microscopic detection of CAMV

The clarified virus concentrate (CVC) was used for the electron microscopic detection of CAMV from young virus infected cowpea leaves. The CVC was layered on collodion coated copper grids and were negatively stained by 1 per cent uranyl acetate.

PLATE 28. Detection on CAMV in infected cowpea plants by NCM-ELISA.

A. Antigen dilution 1:10

B. Antigen dilution 1:50

C. Antigen dilution 1:100

D. Antigen dilution 1:1000

1,2,3. Replicates.

4. Sap from healthy plant.

PLATE 29. Detection of CAMV in viruliferous aphids by NCM-ELISA.

A. Extract from 20 aphids

B. Extract from 10 aphids

C. Extract from 5 aphids

D. Extract from 1 aphid.

1,2,3. Replicates.

4. Extract from non-viruliferous aphids.

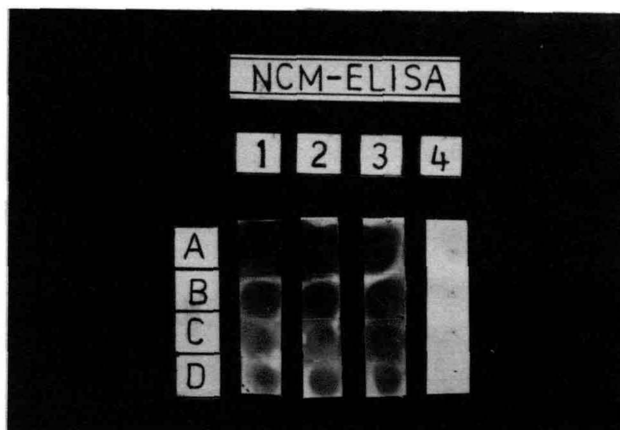



PLATE 28

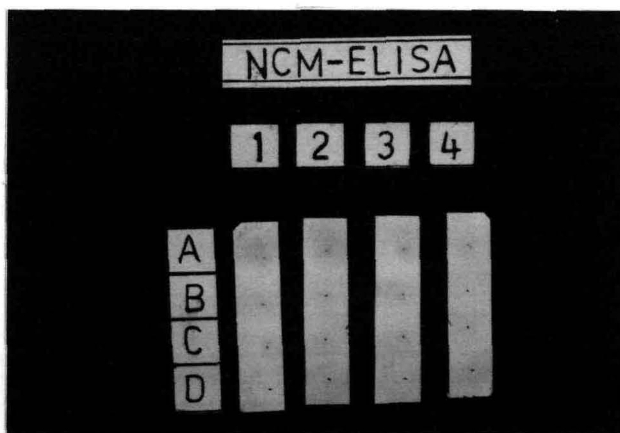



PLATE 29

The grids were examined in a JEOL JEM 100SX transmission electron microscope (Plate 30).

4.6.4 Immunosorbent electron microscopy of CAMV

Immune electron microscopy was performed as outlined by Milne and Luisoni (1977) with slight modification. Antisera to CAMV and potyvirus group antisera were used to trap the virus isolate. The results indicated that both the antisera trapped the virus heavily (Plates 31 and 32).

## 4.7 PHYSIOLOGY AND BIOCHEMISTRY OF VIRUS INFECTED PLANTS

### 4.7.1 Estimation of total chlorophyll

The total chlorophyll contents of the susceptible and resistant cowpea cultivars were estimated as per the procedure described by Arnon (1949) and the results indicated that the total chlorophyll contents of healthy leaves of susceptible and resistant genotypes were significantly greater when compared to the inoculated (Fig. 2). The chlorophyll contents progressively increased in both healthy, resistant (CO 6) and susceptible (C 152 and CO 4) genotypes while there was significant reduction in chlorophyll content in inoculated susceptible varieties. The maximum reduction was observed at 40 days after inoculation. The resistant variety CO 6 did not show much variation due to inocu-

PLATE 30. Electron micrograph of a negatively stained clarified virus concentrate showing flexuous, filamentous CAMV particles $\approx$ 750 nm.

PLATE 31. ISEM electron micrograph of trapped CAMV particles using polyclonal antisera.

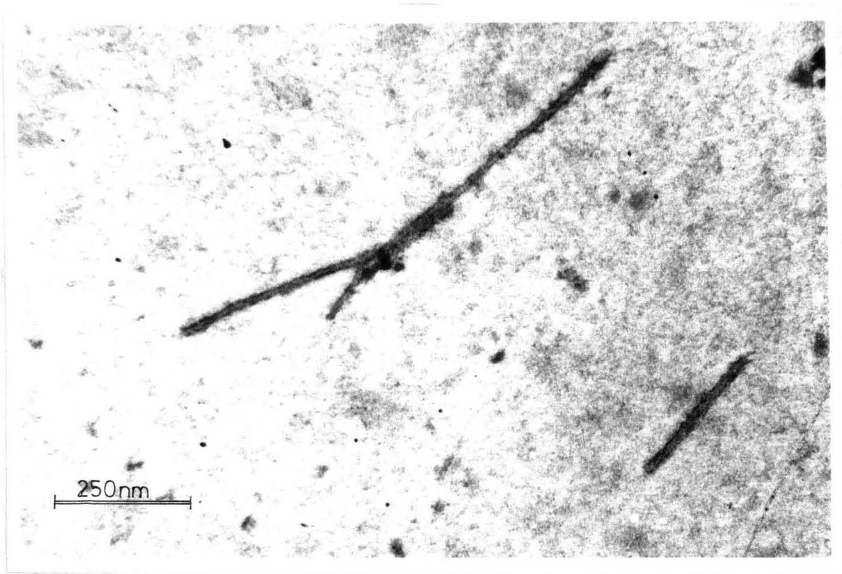



PLATE 31

PLATE 32. ISEM electron micrograph of trapped CAMV particles using potyvirus group antisera.

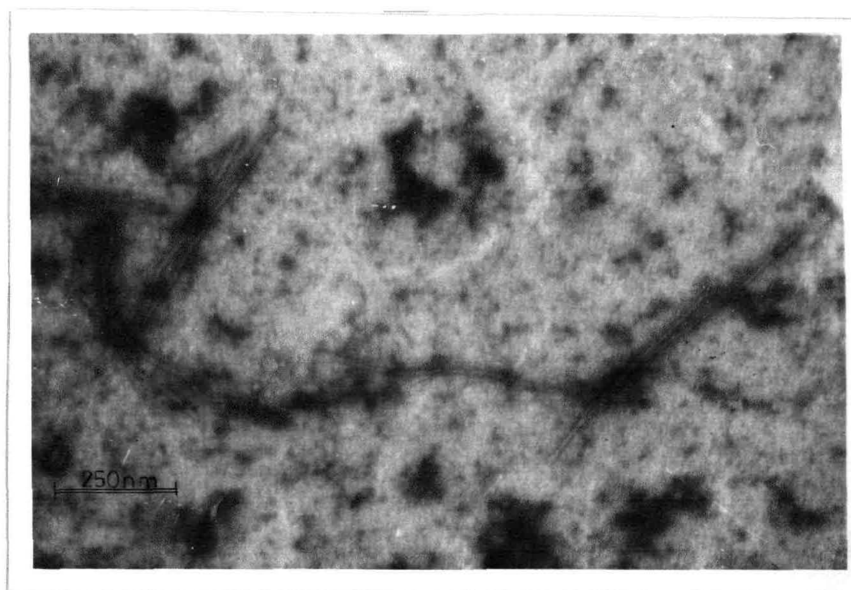
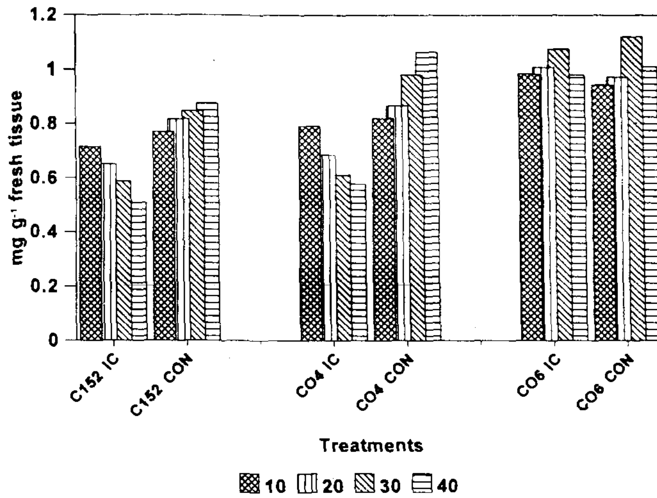



PLATE 32

Fig.2 Changes in total chlorophyll content of cowpea leaves in response to inoculation with CAMV



lation. The resistant varieties showed higher amounts of total chlorophyll than the susceptible varieties (Table 13).

4.7.2 Estimation of total carbohydrate

The data on the changes in total carbohydrate contents of cowpea leaves of both healthy, susceptible and resistant varieties and their response to CAMV inoculation are presented in Table 14.

The results indicated that there was a progressive increase in total carbohydrate content of healthy, susceptible and resistant varieties with the increase in age of the plants. The level of carbohydrate content was significantly lower in susceptible varieties inoculated with CAMV when compared to healthy control. There was also gradual increase in the total carbohydrate content with plant age upon inoculation of susceptible varieties. The resistant variety CO 6 showed higher content of total carbohydrate than the susceptible varieties compared. The resistant variety also showed a reduced level of carbohydrate on inoculation with CAMV but higher than the inoculated and uninoculated susceptible varieties (Fig. 3).

Table 13. Changes in total chlorophyll content of cowpea leaves in response to inoculation with CAMV

| Treatments | | Changes in chlorophyll content (mg $g^{-1}$ fresh weight of tissue) at different days after inoculation | | | |
|---|---|---|---|---|---|
| | | 10 | 20 | 30 | 40 |
| Susceptible varieties: | | | | | |
| C 152 | Inoculated | 0.716 | 0.651 | 0.588 | 0.507 |
| | Control | 0.771 | 0.817 | 0.847 | 0.876 |
| CO 4 | Inoculated | 0.792 | 0.686 | 0.612 | 0.578 |
| | Control | 0.821 | 0.867 | 0.982 | 1.065 |
| Resistant variety | | | | | |
| CO 6 | Inoculated | 0.986 | 1.010 | 1.076 | 0.983 |
| | Control | 0.945 | 0.974 | 1.121 | 1.012 |
| | CD (P=0.05) | 0.056 | 0.028 | 0.032 | 0.056 |

Table 14. Changes in total carbohydrate content of cowpea leaves in response to inoculation with CAMV

| Treatments | | Changes in carbohydrate content ($mg\ g^{-1}$ fresh weight of tissue) at different days after inoculation | | | |
|---|---|---|---|---|---|
| | | 10 | 20 | 30 | 40 |
| Susceptible varieties: | | | | | |
| C 152 | Inoculated | 10.30 | 11.27 | 13.57 | 16.33 |
| | Control | 12.17 | 18.13 | 22.93 | 31.80 |
| CO 4 | Inoculated | 10.53 | 11.20 | 16.80 | 21.20 |
| | Control | 13.73 | 15.80 | 21.80 | 29.87 |
| Resistant variety | | | | | |
| CO 6 | Inoculated | 14.10 | 16.87 | 22.97 | 32.70 |
| | Control | 14.90 | 18.27 | 21.30 | 34.67 |
| | CD (P=0.05) | 0.465 | 0.566 | 0.550 | 0.497 |

### 4.7.3 Estimation of soluble proteins

The data on the changes in total soluble protein content are shown in Table 15. The results indicated that there was a progressive increase in total soluble protein content of healthy plants with the increase in plant age. The level of protein content was high in resistant cultivar when compared to susceptible varieties (Fig. 4).

The susceptible varieties showed a significant increase in the level of total protein, when inoculated with CAMV than in uninoculated control. Inoculation of resistant variety CO 6 with CAMV did not cause much difference in total protein content.

### 4.7.4 Electrophoretic separation of soluble proteins

#### 4.7.4.1 Electrophoretic analysis of seed proteins by SDS-PAGE

Changes in the soluble proteins of seeds of resistant and susceptible cultivar are illustrated in Plate 33.

Differences between healthy and diseased seeds were observed in the fastest migrating bands. Most of these proteins were relatively with low molecular weight. Ten protein bands of molecular weights viz., 26.5, 25.5, 25, 24.5, 24, 18, 17, 15, 14 and 13.7 kDa were observed to be prominent in all the samples

Table 15. Changes in total soluble protein content of cowpea leaves in response to inoculation with CAMV

| Treatments | | Changes in soluble protein content ($mg\ g^{-1}$ fresh weight of tissue) at different days after inoculation | | | |
|---|---|---|---|---|---|
| | | 10 | 20 | 30 | 40 |
| Susceptible varieties: | | | | | |
| C 152 | Inoculated | 0.758 | 0.928 | 1.498 | 1.683 |
| | Control | 0.737 | 0.859 | 1.363 | 1.555 |
| CO 4 | Inoculated | 0.579 | 0.752 | 0.962 | 1.162 |
| | Control | 0.641 | 0.750 | 0.890 | 0.894 |
| Resistant variety | | | | | |
| CO 6 | Inoculated | 0.908 | 1.264 | 1.482 | 1.782 |
| | Control | 0.893 | 1.244 | 1.417 | 1.815 |
| | CD (P=0.05) | 0.016 | 0.016 | 0.016 | 0.016 |

Fig.4 Changes in total soluble protein content of cowpea leaves in response to inoculation with CAMV

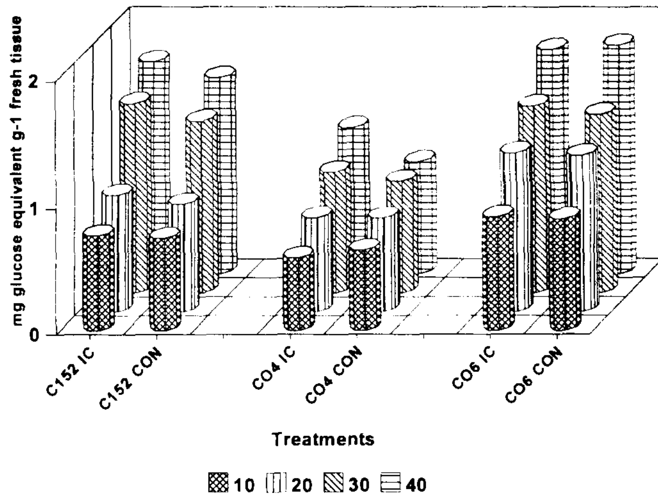


PLATE 33. SDS-PAGE separation of soluble proteins of healthy and infected seeds.

R1 and R2 - Replicates.

Lane 1. Healthy CO4
Lane 2. CAMV infected CO4
Lane 3. CAMV infected C152
Lane 4. Healthy COVU2
Lane 5. Healthy CO6
Lane 6. CAMV infected COVU2.

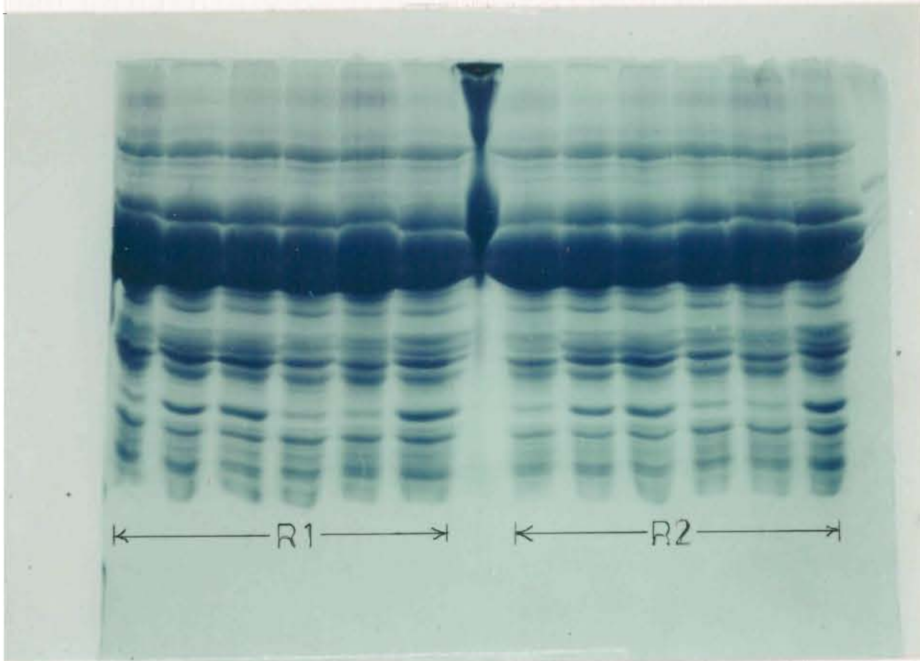



PLATE 33

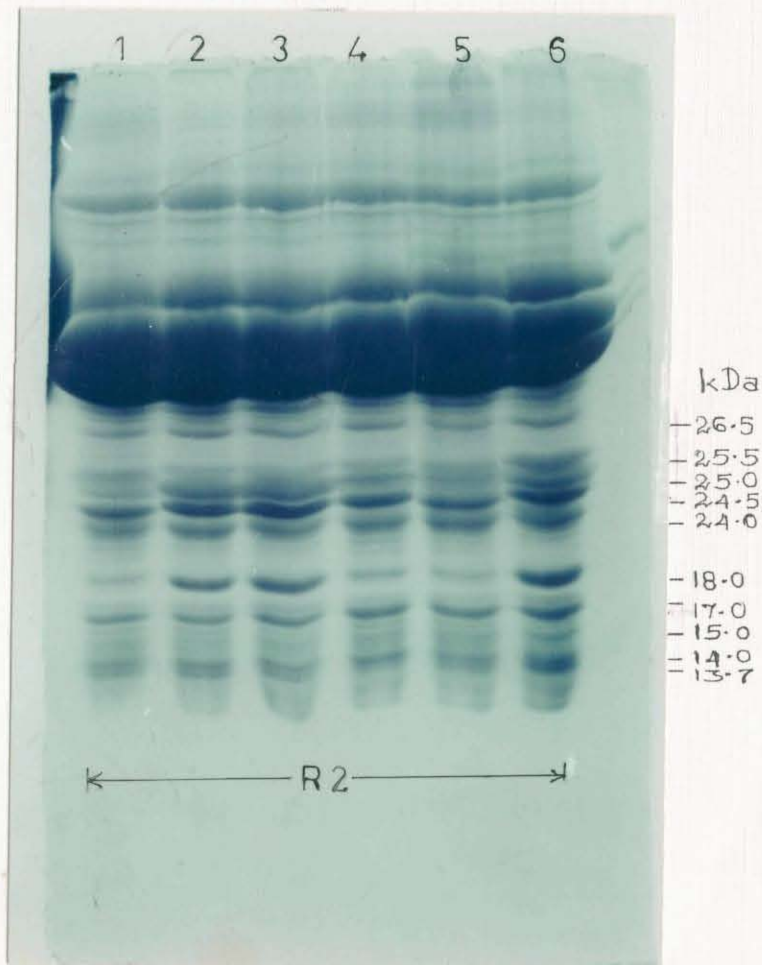



PLATE 33

tested. Among the major protein bands, the 18 kDa was found to be more active and denser in all seed samples collected from diseased plants. The same bands were faint in healthy seed samples from susceptible and resistant lines.

4.7.4.2 Electrophoretic analysis of PR-proteins by SDS-PAGE

Following CAMV inoculation and application of AVPs to cowpea leaves, reproducing alterations to the proteins in the soluble fraction were noted (Plate 34). The denaturing gel electrophoresis of cowpea plants following treatment with AVPs and inoculation with CAMV indicated that at least 9 polypeptides with molecular weights of 34, 31, 30, 25.5, 24, 23, 18, 16 and 13 kDa were induced or stimulated more prominently when compared to control plants. Three novel protein fractions in soluble protein extracts with molecular weight 34, 31 and 23 kDa were detectable in CO 4 cultivar following treatment with AVPs and inoculation with CAMV. The bands were more prominent in 2 days after inoculation (lane 1) and 2 days after spraying with AVPs in combination with 1 day after inoculation (lane 3). No differences were observed between the electrophoretic protein profiles of samples from healthy untreated control plants (lanes 5, 6 and 7).

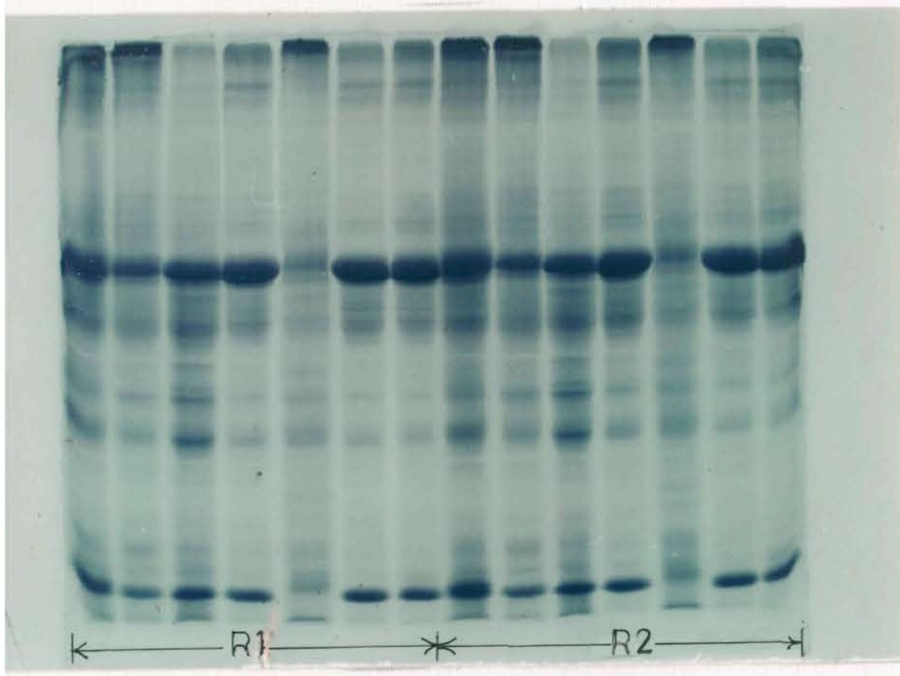



PLATE 34

PLATE 34

4.7.4.3 Electrophoretic analysis of leaf isozymes by non-denaturing gel electrophoresis

The electrophoretic patterns of peroxidase isozymes (isoPOs) stained by benzidine are shown in Plate 35. Soluble protein fractions of the genotypes which contained isoPOs occupied the first half of the gel. Differences between the genotypes were observed in the migrating bands. The Rf (distance of migration of a given band relative to the distance of migration of the bromophenol blue marker dye) values of each band were calculated and relative mobility were diagramatically represented in Fig. 5. The Rfs and relative intensities of the isozyme bands of peroxidase were also recorded (Table 16).

Atleast three isoPOs in the four susceptible genotypes at Rf values of 0.07, 0.33 and 0.71 in C 152; 0.13, 0.33 and 0.71 in CO 4; 0.13, 0.33 and 0.45 in CO 2; 0.13, 0.33 and 0.71 in CoVU-2 could be identified with that banding at Rf 0.13 in CoVU-2 and 0.33 in CO 2 and CoVU-2 being the most active compared to other bands. Lanes 5 to 10 represents the isoPOs in six resistant genotypes. Three isoforms of peroxidase enzymes at Rf values 0.33, 0.45 and 0.71 were detected in all the six resistant genotype with particular enhancement to the isozyme band at Rf 0.33. The activity of this enzyme was substantially higher than the other isoPOs in the gel. One apparently novel isozyme was

PLATE 35. Native PAGE pattern showing peroxidase isozymes from leaf extracts of 10 cowpea genotypes.

| Lane | Genotype | Reaction |
| --- | --- | --- |
| Lane 1. | C152 | Susceptible to CAMV |
| Lane 2. | CO4 | |
| Lane 3. | CO2 | |
| Lane 4. | COVU2 | |
| Lane 5. | NBPGR-759 | Resistant to CAMV |
| Lane 6. | NBPGR-784 | |
| Lane 7. | NBPGR-1164 | |
| Lane 8. | NBPGR-1183 | |
| Lane 9. | NBPGR-1195 | |
| Lane 10. | CO6 | |

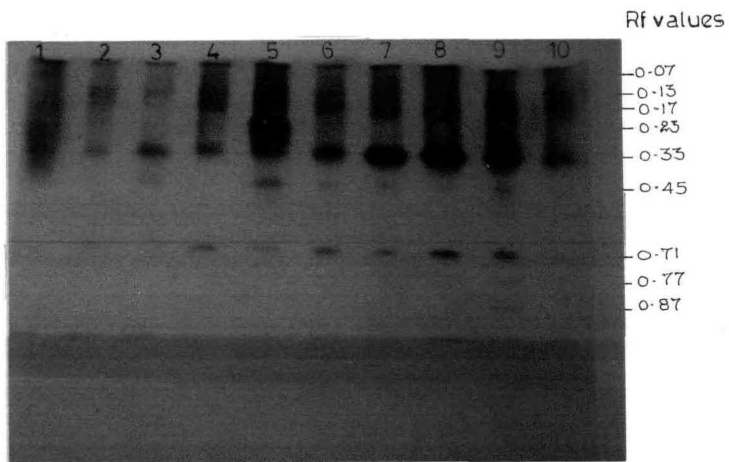



PLATE 35

Fig. 5. Diagrammatic representation of peroxidase isozyme in leaf extracts from 10 cowpea genotypes showing differential reaction to CAMV.

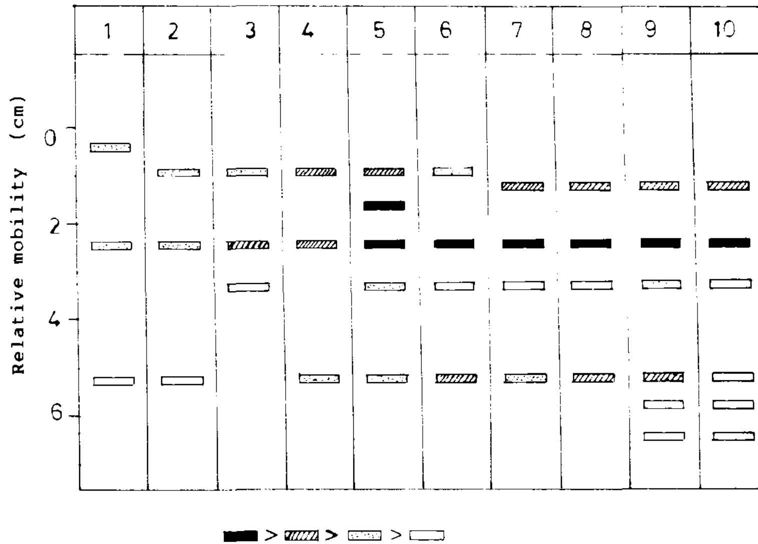


**Table 16.** The Rfs and relative intensities@ of isozyme bands of peroxidase in 10 genotypes of cowpea showing differential reaction to CAMV

| Rf* | Genotypes | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | C 152 | CO 4 | CO 2 | CoVU-2 | NBPGR 759 | NBPGR 784 | NBPGR 1164 | NBPGR 1183 | NBPGR 1195 | CO 6 |
| 0.07 | XX | | | | | | | | | |
| 0.13 | | XX | XX | XXX | XXX | XX | | | | |
| 0.17 | | | | | | | XXX | XXX | XXX | XXX |
| 0.23 | | | | | XXXX | | | | | |
| 0.33 | XX | XX | XXX | XXX | XXXX | XXXX | XXXX | XXXX | XXXX | XXXX |
| 0.45 | | | X | | XX | X | X | X | XX | X |
| 0.71 | X | X | | XX | XX | XXX | XX | XXX | XXX | X |
| 0.77 | | | | | | | | | X | X |
| 0.87 | | | | | | | | | X | X |

$$* Rf = \frac{\text{distance that enzyme band migrates}}{\text{distance that marker dye migrates}}$$

@ Increasing intensity of bands are represented X to XXXX

detected at Rf 0.45 for the six resistant genotypes. In addition to the three common bands shared by the resistant lines, the genotype NBPGR 759 had two more additional bands of isoPOs at Rf 0.13 and 0.23, where the band at Rf 0.23 was more active. The genotypes NBPGR 1195 and CO 6 also exhibited two additional bands at Rf 0.77 and 0.87 but the density of the band was very faint when compared to other bands obtained in the experiment.

#### 4.7.5 Estimation of nucleic acids

The changes in nucleic acid contents of susceptible and resistant genotypes were studied following inoculation with CAMV. Changes in RNA and DNA contents were assessed at 10, 20, 30 and 40 days after inoculation.

##### 4.7.5.1 Estimation of ribonucleic acid (RNA)

The results in Table 17 indicated that there was significant alterations in RNA content in susceptible cultivars following inoculation with CAMV. The RNA content of healthy plants of both resistant and susceptible cultivars was found to increase with increase in plant age. There was a gradual increase in RNA content in susceptible cultivars following inoculation with CAMV. The increase was maximum at 30 days after inoculation and thereafter it showed a decreasing trend, when samples

Table 17. Changes in RNA contents of cowpea leaves in response to inoculation with CAMV

| Treatments | | Changes in RNA contents ($\mu$ mole $g^{-1}$ fresh weight of tissue) at different days after inoculation | | | |
|---|---|---|---|---|---|
| | | 10 | 20 | 30 | 40 |
| Susceptible varieties: | | | | | |
| C 152 | Inoculated | 1.563 | 1.592 | 1.623 | 1.609 |
| | Control | 1.536 | 1.552 | 1.568 | 1.573 |
| CO 4 | Inoculated | 1.680 | 1.695 | 1.722 | 1.713 |
| | Control | 1.612 | 1.617 | 1.636 | 1.642 |
| Resistant variety | | | | | |
| CO 6 | Inoculated | 1.838 | 1.872 | 1.878 | 1.885 |
| | Control | 1.851 | 1.865 | 1.872 | 1.896 |
| | CD (P=0.05) | 0.016 | 0.016 | 0.016 | 0.016 |

were analysed at 40 days after inoculation. The RNA content of inoculated susceptible cultivars tested maintained at a higher level when compared to uninoculated control. The levels of RNA in resistant cultivar CO 6 was found to be higher in both inoculated and healthy plants when compared to susceptible cultivars. Inoculation with CAMV did not alter the RNA content in the resistant cultivar (Fig. 6).

4.7.5.2 Deoxyribonucleic acid (DNA)

The results on the changes in DNA content are presented in Table 18. The data indicated that the DNA content in resistant cultivar was higher than in susceptible cultivars. The level of DNA content was low in inoculated susceptible cultivars when compared to uninoculated control (Fig.7). There was a gradual increase in the level of DNA content in both susceptible and resistant cultivar except in CO 4 where the maximum amount of DNA was observed at 30 days after inoculation and the level showed a decreasing trend when the sample was analysed 40 days after inoculation. The resistant cultivar CO 6 did not show any significant variation in the content of DNA following inoculation.

**Fig.6 Changes in RNA content of cowpea leaves in response to inoculation with CAMV**

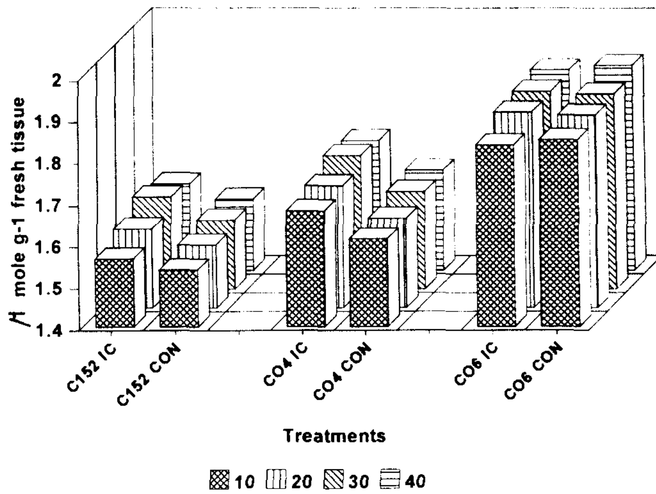
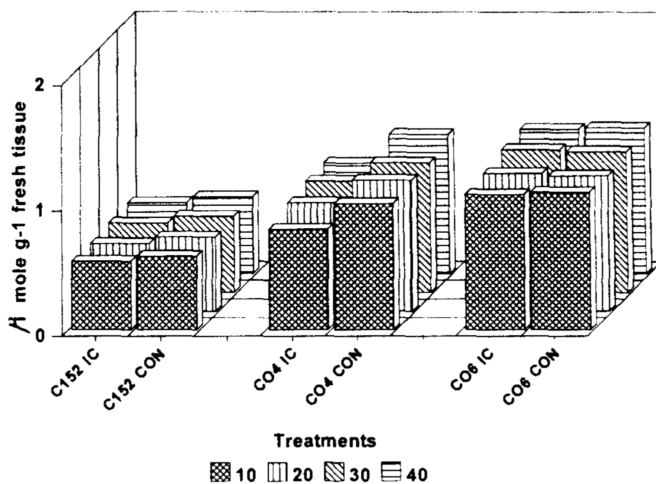


Table 18. Changes in DNA contents of cowpea leaves in response to inoculation with CAMV

| Treatments | | Changes in DNA contents ($\mu$ mole $g^{-1}$ fresh weight of tissue) at different days after inoculation | | | |
|---|---|---|---|---|---|
| | | 10 | 20 | 30 | 40 |
| Susceptible varieties: | | | | | |
| C 152 | Inoculated | 0.548 | 0.538 | 0.554 | 0.565 |
| | Control | 0.589 | 0.592 | 0.608 | 0.613 |
| CO 4 | Inoculated | 0.805 | 0.866 | 0.890 | 0.885 |
| | Control | 1.010 | 1.048 | 1.038 | 1.113 |
| Resistant variety | | | | | |
| CO 6 | Inoculated | 1.082 | 1.104 | 1.143 | 1.155 |
| | Control | 1.101 | 1.085 | 1.125 | 1.167 |
| | CD (P=0.05) | 0.022 | 0.022 | 0.016 | 0.016 |

**Fig.7 Changes in DNA content of cowpea leaves in response to inoculation with CAMV**



### 4.7.6 Assay of enzymes

#### 4.7.6.1 Peroxidase activity

The results (Table 19) indicated that the peroxidase activity increased progressively upto five days after inoculation and there was a decline in the activity in samples taken at 10 and 15 days after treatment in resistant and susceptible cultivars. The increase was 44.41 per cent more than the uninoculated control in plant samples at 5 days after inoculation in the susceptible variety. The level of peroxidase activity was also found to be 46.22 per cent when the susceptible variety was inoculated after treatment with *Bougainvillea spectabilis* leaf extract (BSLE). The enzyme activity was enhanced even with BSLE treatment alone, which showed a 38.37 per cent more than the untreated control.

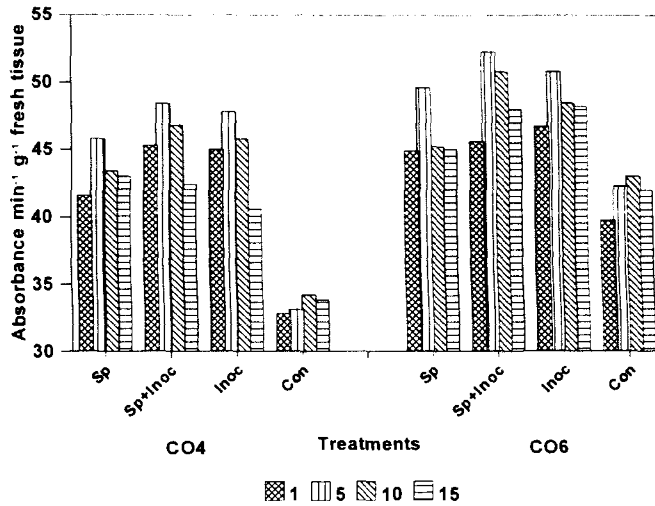
The resistant variety CO 6 also showed the same trend of enzyme activity but the change in absorbance was more when compared to susceptible sample analysed. The induction of enzyme activity was more in all treatments given. The activity showed a declining trend in samples taken at 10 and 15 days after treatment (Fig. 8).

Table 19. Changes in peroxidase activity in cowpea plants in response to inoculation with CAMV and BSLE treatment

| Treatment | Peroxidase activity (Changes in absorbance $min^{-1}$ $g^{-1}$ fresh weight) at different days after treatment | | | |
|---|---|---|---|---|
| | 1 | 5 | 10 | 15 |
| **Susceptible variety CO 4** | | | | |
| i) Sprayed (with BSLE) | 41.600 (+26.83) | 45.800 (+38.37) | 43.400 (+26.90) | 43.000 (+27.09) |
| ii) Sprayed + Inoculated | 45.300 (+38.11) | 48.400 (+46.22) | 46.800 (+36.84) | 42.400 (+25.32) |
| iii) Inoculated | 45.500 (+38.72) | 47.800 (+44.41) | 45.800 (+33.92) | 40.600 (+20.00) |
| iv) Control | 32.800 | 33.100 | 34.200 | 33.833 |
| **Resistant variety CO 6** | | | | |
| i) Sprayed (with BSLE) | 44.900 (+12.81) | 49.600 (+17.26) | 45.200 (+ 5.12) | 45.000 (+ 7.14) |
| ii) Sprayed + Inoculated | 45.600 (+14.57) | 52.300 (+23.64) | 50.800 (+18.14) | 48.000 (+14.29) |
| iii) Inoculated | 46.700 (+17.34) | 50.800 (+20.09) | 48.433 (+12.64) | 48.200 (+14.76) |
| iv) Control | 39.800 | 42.300 | 43.000 | 42.000 |
| CD (P=0.05) Treatments | 1.462 | 1.741 | 2.055 | 2.011 |
| Days | 1.034 | 1.231 | 1.453 | 1.422 |
| Varieties | 0.731 | 0.870 | 1.028 | 1.006 |

Figures in parenthesis represent per cent increase (+) over healthy (Control)

**Fig.8 Changes in peroxidase activity in cowpea plants in response to inoculation with CAMV and BSLE treatment**



### 4.7.6.2 Polyphenol oxidase activity

The polyphenol oxidase (PPO) activity showed an increasing trend in both susceptible and resistant cultivars with increase in age of the plants. The PPO activity in susceptible cultivar CO 4 attained its peak at 10 days after treatment and thereafter it declined. The maximum activity was observed when the plants were sprayed and inoculated. There was an increase of 57.61 per cent than the healthy control (Table 20).

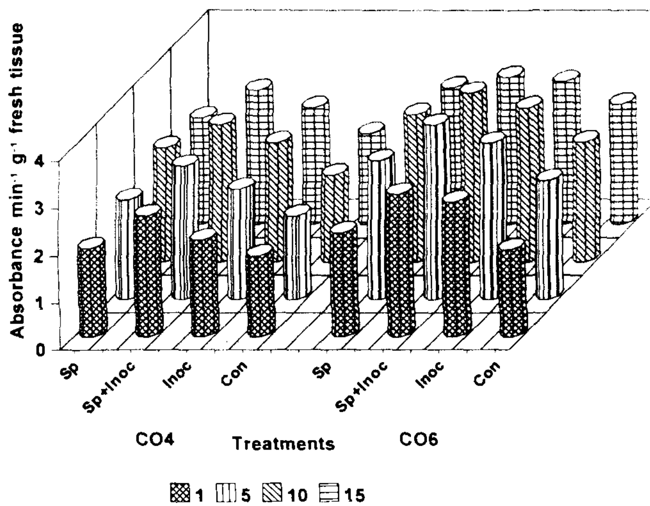
The PPO in resistant variety CO 6 also showed a high level of activity in treated plants when compared to the control. The induction of the enzyme activity was more at one day after all treatments and reached a maximum at 5 days after inoculation with CAMV and combined treatment (BSLE spray + inoculation). The peak enzyme activity on BSLE treatment was observed 10 days after treatment. The PPO activity increased significantly at all periods of analysis when compared to the susceptible variety under treatments. Inoculation of BSLE treated resistant plants showed the maximum PPO activity. The maximum per cent increase of 68.33 was observed in plants at one day after treatment in the resistant line tested (Fig. 9).

Table 20. Changes in polyphenol oxidase activity in cowpea plants in response to inoculation with CAMV and BSLE treatment

| Treatment | Polyphenol oxidase activity (Changes in absorbance $min^{-1}$ $g^{-1}$ *fresh weight*) at different days after treatment | | | |
|---|---|---|---|---|
| | 1 | 5 | 10 | 15 |
| Susceptible variety CO 4 | | | | |
| i) Sprayed (with BSLE) | 1.887 (+ 9.08) | 2.100 (+18.64) | 2.410 (+30.98) | 2.240 (+16.67) |
| ii) Sprayed + Inoculated | 2.570 (+48.56) | 2.820 (+59.32) | 2.900 (+57.61) | 2.827 (+47.24) |
| iii) Inoculated | 2.060 (+19.08) | 2.330 (+31.64) | 2.520 (+36.96) | 2.460 (+28.13) |
| iv) Control | 1.730 | 1.770 | 1.840 | 1.920 |
| Resistant variety CO 6 | | | | |
| i) Sprayed (with BSLE) | 2.220 (+23.33) | 2.940 (+16.67) | 3.120 (+22.84) | 2.890 (+13.78) |
| ii) Sprayed + Inoculated | 3.030 (+68.33) | 3.700 (+46.83) | 3.580 (+40.95) | 3.120 (+22.84) |
| iii) Inoculated | 2.870 (+59.44) | 3.320 (+31.75) | 3.260 (+28.35) | 3.020 (+18.90) |
| iv) Control | 1.800 | 2.520 | 2.540 | 2.540 |
| CD (P=0.05) Treatment | 0.235 | 0.266 | 1.588 | 0.166 |
| Days | 0.166 | 0.188 | 1.123 | 0.118 |
| Varieties | 0.118 | 0.133 | 0.794 | 0.083 |

Figures in parentheses represent per cent increase (+) over healthy (control)

Fig.9 Changes in polyphenol oxidase activity in cowpea plants in response to inoculation with CAMV and BSLE treatment



4.7.6.3 Phenylalanine ammonia-lyase (PAL) activity

Studies on the changes in phenylalanine ammonia-lyase (PAL) was carried out to elucidate its alterations in cowpea plants in response to inoculation with CAMV and treatment with BSLE. The results of the experiments are presented in Table 21.

The observation showed that there was an increasing trend in the PAL activity in uninoculated control of both susceptible and resistant cultivars, which was in proportion to the age of the plant. The PAL activity in the resistant plant was found to be greater when compared to the susceptible variety at all periods of analysis.

Susceptible plants on inoculation showed a significant increase in the PAL activity upto 10 days after treatment and thereafter it showed a decreasing trend. Inoculation of CO 4 with CAMV showed a maximum increase of 72.34 per cent PAL activity at 10 days after inoculation when compared to healthy control plants. The activity of PAL was at its peak in samples analysed from plants inoculated after treatments with BSLE application after 10 days. There was an increase of 117.01 per cent. Treatment of CO 4 variety with BSLE also resulted in an increase of 31.97 per cent over control at 10 days after application where

Table 21. Changes in phenylalanine ammonia lyase (PAL) activity in cowpea plants in response to inoculation with CAMV and BSLE treatment

| Treatments | Changes in PAl activity (n mole trans cinnamic acid $h^{-1}$ $g^{-1}$ fresh weight) at different days after treatment | | | |
|---|---|---|---|---|
| | 1 | 5 | 10 | 15 |
| **Susceptible variety CO 4** | | | | |
| i) Sprayed (with BSLE) | 862.333<br>(+5.94) | 1059.000<br>( +19.39) | 1288.000<br>(+ 31.97) | 872.333<br>(-28.79) |
| ii) Sprayed + Inoculated | 845.000<br>(+3.81) | 1880.000<br>(+111.95) | 2118.000<br>(+117.01) | 1828.000<br>(+49.22) |
| iii) Inoculated | 824.000<br>(+1.23) | 1298.333<br>(+ 46.37) | 1682.000<br>(+ 72.34) | 1360.000<br>(+11.02) |
| iv) Control | 814.000 | 887.000 | 976.000 | 1225.000 |
| **Resistant variety CO 6** | | | | |
| i) Sprayed (with BSLE) | 882.000<br>(-1.23) | 1724.000<br>( +90.92) | 1910.000<br>(+ 87.62) | 1329.333<br>(+ 3.18) |
| ii) Sprayed + Inoculated | 866.667<br>(-2.95) | 2284.000<br>(+152.94) | 2741.000<br>(+169.25) | 2284.000<br>(+77.33) |
| iii) Inoculated | 884.000<br>(-1.01) | 1900.000<br>(+110.41) | 2336.000<br>(+129.47) | 1786.000<br>(+38.67) |
| iv) Control | 893.000 | 903.000 | 1018.000 | 1288.000 |
| CD (P=0.05) Treatment | 11.274 | 12.429 | 12.455 | 14.586 |
| Days | 7.972 | 8.789 | 8.807 | 10.314 |
| Varieties | 5.637 | 6.215 | 6.227 | 7.293 |

Figures in parentheses represent per cent increase (+) or decrease (-) over healthy (control)

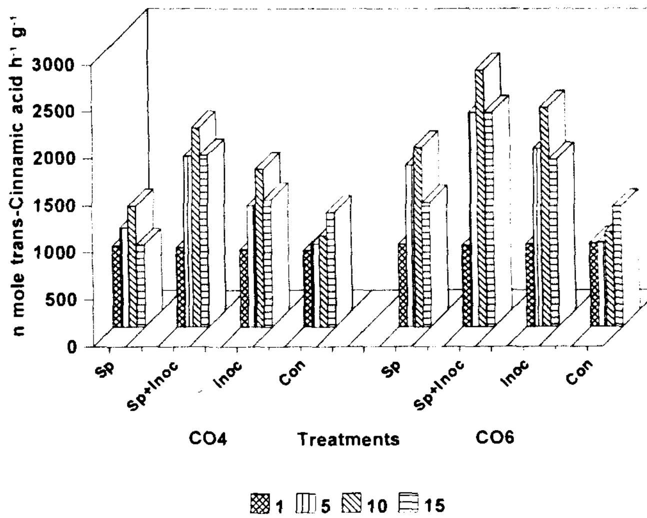
the activity was found to be less when compared to other two treatments.

The resistant variety CO 6 also showed the same trend but the level of enzyme activity was significantly higher than the susceptible line tested. The activity of PAL was at its maximum (129.47 per cent) when inoculated with CAMV at 10 days after inoculation in comparison with its uninoculated control. Inoculation of BSLE treated plants also showed a maximum level of PAL activity of 169.25 per cent more in leaf samples analysed 10 days after treatment when compared to healthy plants. The PAL activity showed a decreasing trend in samples taken at 15 days after different treatments (Fig. 10).

#### 4.7.6.4 Chitinase activity

Substantial increase in chitinase activity was observed in the treated susceptible and resistant cultivars. There was only gradual increase in the level of enzyme in the healthy control of both the cultivars. The chitinase activity increased in the leaves of the test plants from 2 days after inoculation with the pathogen and in plants inoculated after treatment with BSLE or treatment of BSLE alone. The maximum increase in activity was observed at 8 days after inoculation in both resistant and susceptible lines. Samples analysed at 10 days after various

**Fig.10 Changes in phenylalanine ammonia-lyase activity in cowpea plants in response to inoculation with CAMV and BSLE treatment**



treatments showed a significant reduction in the enzyme activity (Table 22).

Increased chitinase activity was consistently observed in leaves inoculated with the pathogen in susceptible CO 4 cultivar. Inoculation of BSLE treated plants showed a significant increase in the level of enzyme activity when compared to that of other two treatments in CO 4 (5.78, 7.163, 9.04, 9.290 and 8.673 $\mu m\ h^{-1}\ g^{-1}$ fresh weight of 2, 4, 6, 8 and 10 days after tretment respectively). The enzyme showed its peak activity at 8 days after treatment. The same trend was observed on BSLE treatment.

Following different treatments on the resistant cultivar CO 6, there was substantial increase in the level of chitinase activity which was significantly higher when compared to susceptible cultivar. The activity increased progressively and the enzyme showed its maximum level at 8 days after different treatments and thereafter it showed a decline. There was marked differences between susceptible and resistant lines in the enzyme activity at all periods of analysis (Fig. 11).

#### 4.7.7 Estimation of phenolics

##### 4.7.7.1 Total phenols

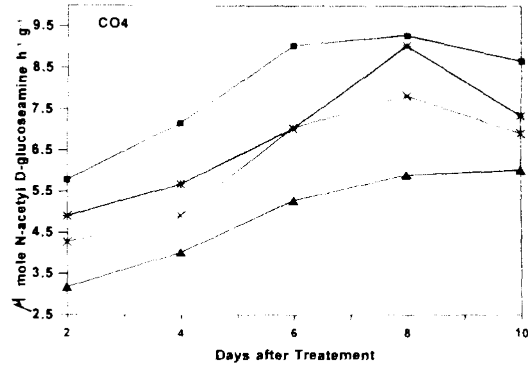
The total phenol content increased with increase in age of both resistant (CO 6) and susceptible (C 152 and CO 4) healthy

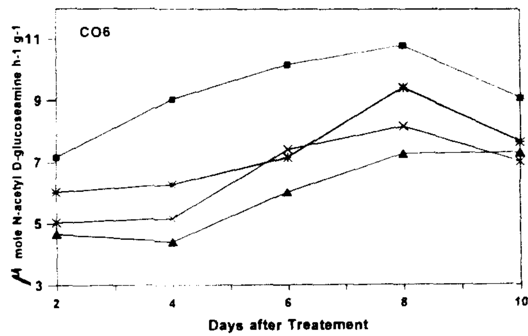
Table 22. Changes in chitinase activity in cowpea plants in response to inoculation with CAMV and BSLE treatment

| Treatment | Chitinase activity ($\mu$ mole N-acetyl glucoseamine $h^{-1}$ $g^{-1}$ fresh weight at different days after treatment | | | | |
|---|---|---|---|---|---|
| | 2 | 4 | 6 | 8 | 10 |
| <u>Susceptible variety CO 4</u> | | | | | |
| i) Sprayed (with BSLE) | 4.900 (+54.57) | 5.680 (+ 41.29) | 7.027 (+33.09) | 9.040 (+53.22) | 7.340 (+62.28) |
| ii) Sprayed + Inoculated | 5.780 (+82.33) | 7.163 (+ 78.18) | 9.040 (+71.21) | 9.290 (+57.46) | 8.673 (+91.75) |
| iii) Inoculated | 4.270 (+34.70) | 4.920 (+ 22.39) | 7.040 (+33.33) | 7.830 (+32.71) | 6.910 (+14.73) |
| iv) Control | 3.170 | 4.020 | 5.280 | 5.900 | 6.023 |
| <u>Resistant variety CO 6</u> | | | | | |
| i) Sprayed (with BSLE) | 6.030 (+29.68) | 6.280 (+ 42.73) | 7.160 (+18.74) | 9.420 (+29.34) | 7.657 (+24.50) |
| ii) Sprayed + Inoculated | 7.160 (+53.98) | 9.040 (+105.46) | 10.170 (+68.66) | 10.800 (+48.29) | 9.093 (+47.85) |
| iii) Inoculated | 5.033 (+ 8.24) | 5.160 (+ 17.27) | 7.410 (+22.89) | 8.160 (+12.04) | 7.030 (- 3.90) |
| iv) Control | 4.650 | 4.400 | 6.030 | 7.283 | 7.315 |
| CD(P=0.05) Treatment | 0.124 | 0.200 | 0.110 | 0.078 | 0.055 |
| Days | 0.088 | 0.141 | 0.068 | 0.055 | 0.039 |
| Varieties | 0.062 | 0.100 | 0.048 | 0.039 | 0.028 |

Figures in parentheses represent per cent increase (+) or decrease (-) over healthy (control)

**Fig.11 Changes in chitinase activity in cowpea plants in response to inoculation with CAMV and BSLE treatment**




Sp — Sp+Inoc — Inoc — Con

control plants. The resistant line showed a significantly higher total phenol content when compared to the susceptible lines at all stages of analysis (Table 23).

Phenolic content increased in leaves of susceptible lines even three days after inoculation with the pathogen. Contrastingly there was a decrease from 4 days after inoculation. The total phenol content reached its maximum of 1216.67 µg and 1372.67 µg/g fresh weight of tissue in C 152 and CO 4 cultivars, which were 5.10 and 22.85 per cent higher than the healthy control respectively.

The total phenol content was consistently more throughout the experimental period in leaves of resistant variety after inoculation with the pathogen. The increase was maximum at 3 days after inoculation. The total phenol content was 1580.33 µg/g leaf tissue, which was 24.21 per cent more than the healthy control. There was a marked decrease in its content in leaf samples analysed at 4 and 5 days after inoculation (Fig. 12).

4.7.7.2 Ortho-dihydroxy (OD) phenols

The observation on the changes in OD phenols also indicated that there was an increase in OD phenol content with an increase in plant age, in both susceptible and resistant cultivar (Table 24).

Table 23. Changes in total phenol content of cowpea leaves in response to inoculation with CAMV

| Treatments | | Changes in total phenol content (µg/g fresh weight of tissue) at different days after inoculation | | | | |
|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 |
| Susceptible varieties | | | | | | |
| 1. C 152 | Inoculated | 1139.667 (+ 2.55) | 1197.000 (+ 3.82) | 1216.667 (+ 5.10) | 1149.667 (- 1.85) | 1085.333 (- 8.00) |
| | Control | 1111.333 | 1153.000 | 1157.667 | 1171.333 | 1179.667 |
| 2. CO 4 | Inoculated | 1113.333 (+ 2.52) | 1226.667 (+10.38) | 1372.667 (+22.85) | 1060.667 (- 4.96) | 977.000 (-12.77) |
| | Control | 1086.000 | 1111.333 | 1117.333 | 1116.000 | 1120.667 |
| Resistant variety | | | | | | |
| 3. CO 6 | Inoculated | 1493.333 (+19.75) | 1542.000 (+22.38) | 1580.333 (+24.21) | 1385.000 (+ 5.56) | 1382.000 (+ 4.59) |
| | Control | 1247.000 | 1260.000 | 1272.333 | 1312.000 | 1321.333 |
| CD (P = 0.05) | | 6.472 | 8.819 | 8.290 | 8.254 | 13.298 |

Figures in parentheses represent per cent increase (+) or decrease (-) over healthy (control)

**Fig.12 Changes in total phenol content of cowpea leaves in response to inoculation with CAMV**

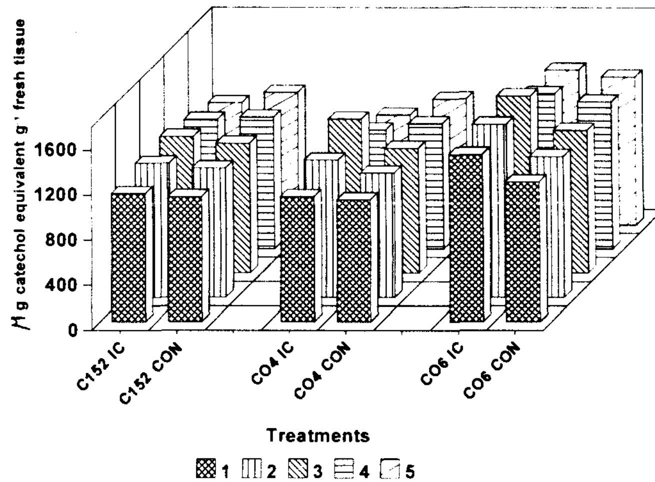


Table 24. Changes in O-dihydroxyphenol content of cowpea leaves *in response to inoculation with* CAMV

| Treatments | | Changes in O-dihydroxyphenol (µg/g fresh weight of tissue) at different days after inoculation | | | | |
|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 |
| <u>Susceptible varieties</u> | | | | | | |
| 1. C 152 | Inoculated | 133.600<br>(+ 8.71) | 147.133<br>(+18.53) | 160.000<br>(+26.18) | 141.700<br>(+10.62) | 132.600<br>(+ 1.84) |
| | Control | 122.900 | 124.133 | 126.800 | 128.100 | 130.200 |
| 2. CO 4 | Inoculated | 122.900<br>(+24.65) | 130.933<br>(+23.29) | 163.200<br>(+50.28) | 112.100<br>(+ 1.48) | 101.300<br>(-11.30) |
| | Control | 98.600 | 106.200 | 108.600 | 110.467 | 114.200 |
| <u>Resistant variety</u> | | | | | | |
| 3. CO 6 | Inoculated | 171.300<br>(+25.68) | 174.000<br>(+26.00) | 187.400<br>(+32.31) | 176.100<br>(+22.12) | 168.900<br>(+15.45) |
| | Control | 136.300 | 138.100 | 141.633 | 144.200 | 146.300 |
| CD (P = 0.05) | | 1.494 | 1.273 | 0.970 | 1.437 | 0.926 |

Figures in parentheses represent increase (+) or decrease (-) over healthy (control)

The susceptible plants showed a steady increase in OD phenol content upto 3 days after inoculation, where it reached its maximum and decreased thereafter. A concentration of 160.0 µg/g fresh tissue was observed at its peak, which was 26.18 per cent more in comparison with its control healthy plant of C 152 cultivar. CO 4 showed a maximum of 163.2 µg/g which was 50.28 per cent more than the control.

The OD phenol content was found to be more in both inoculated and control plants of CO 6 variety, in comparison to the susceptible varieties. The OD phenol content reached its peak at 3 days after inoculation. The maximum increase was 187.4 µg/g, which was 32.31 per cent more than the control compared. There was a marked decline in OD phenol content in leaf samples analysed 4 and 5 days after inoculation (Fig. 13).

## 4.8 MANAGEMENT OF CAMV

### 4.8.1 Management of CAMV using virus inhibitory chemicals

A range of inorganic salts at concentration from 250 to 1000 ppm were treated as foliar spray prior to inoculation with CAMV on *C.amaranticolor*. As listed in Table 25, 20 chemicals at different concentrations, showing antiphytoviral activity were tested against CAMV to evaluate their efficacy in inhibition of the disease. The concentrations of the chemicals were fixed after evaluating their phytotoxic level.

**Fig.13 Changes in *ortho* dihydroxy phenol content of cowpea leaves in response to inoculation with CAMV**

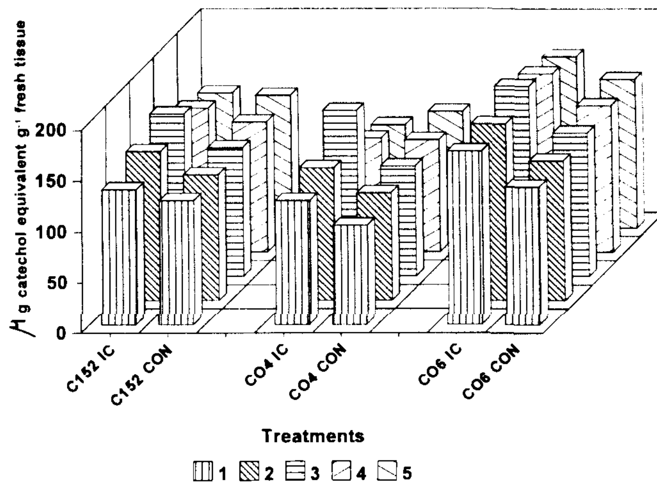


Table 25. Effect of chemicals on local lesion production by CAMV on *Chenopodium amaranticolor*

| Sl. No. | Treatments | Concentration (ppm) | Mean No. of lesions/ $cm^2$ | Inhibition (%) |
|---|---|---|---|---|
| 1. | Ammonium molydate | 1000 | 2.950 | 84.44 |
| 2. | Potassium dihydrogen phosphate | 1000 | 5.491 | 71.03 |
| 3. | Sodium acetate | 1000 | 6.653 | 64.90 |
| 4. | Ascorbic acid | 1000 | 17.663 | 6.81 |
| 5. | Salicylic acid | 1000 | 5.152 | 72.82 |
| 6. | Salicylic acid | 750 | 8.439 | 55.47 |
| 7. | Salicylic acid | 500 | 13.665 | 27.90 |
| 8. | Sodium sulphite | 1000 | 11.913 | 37.15 |
| 9. | Potassium permanganate | 1000 | 8.074 | 57.40 |
| 10. | Magnesium sulphate | 1000 | 16.265 | 14.18 |
| 11. | Copper sulphate | 1000 | 2.988 | 84.24 |
| 12. | Nicotinic acid | 1000 | 7.027 | 62.92 |
| 13. | Copper acetate | 1000 | 14.254 | 24.79 |
| 14. | Manganese sulphate | 1000 | 13.751 | 27.45 |
| 15. | Zinc sulphate | 1000 | 13.616 | 28.16 |
| 16. | Potassium chloride | 1000 | 14.427 | 23.88 |
| 17. | Barium chloride | 1000 | 1.453 | 92.33 |
| 18. | Bavistin | 1000 | 16.049 | 15.32 |
| 19. | Bavistin | 500 | 16.018 | 15.49 |
| 20. | Thiouracil | 500 | 1.916 | 89.89 |
| 21. | Thiouracil | 250 | 4.584 | 75.81 |
| 22. | Aspirin (ASA) | 1000 | 7.694 | 59.41 |
| 23. | Aspirin (ASA) | 500 | 13.575 | 28.38 |
| 24. | Nickel chloride | 500 | 12.481 | 34.15 |
| 25. | Silver nitrate | 250 | 15.135 | 20.15 |
| 26. | Control | - | 18.953 | - |
| | C.D. (P = 0.05) | | 1.254 | |

The results of the experiments indicated that pre-inoculation sprays of the chemicals inhibited the viral infection. The maximum inhibition of 92.33 per cent was obtained with 1000 ppm barium chloride spray. Five hundred ppm concentration of thiouracil, 1000 ppm of ammonium molybdate and copper sulphate gave an inhibition of 89.89, 84.44 and 84.24 per cent respectively.

### 4.8.2 Management of CAMV using plant products

#### 4.8.2.1 Screening of plant species for the presence of antiviral principles (AVPs)

The effectiveness of aqueous leaf extracts from 25 selected plant species were tested for the presence of AVPs against CAMV. Ten per cent concentration of the leaf extracts were sprayed on assay host 24 h prior to inoculation.

The results in Table 26 indicated that all the extracts significantly reduced the CAMV infection, where the inhibition ranged from 32 to 100 per cent. Among the 25 plant species tested, *C.annum* had the highest antiviral activity of 100 per cent. However four leaf extracts from *B.rubra*, *C.bonplandianum*, *I.carnea* and *M.jalapa* were also equally effective in reducing CAMV infection, which gave an inhibition of 99.67 per cent. Among the species screened others which reduced the infection by more than 90 per cent were *B.spectabilis* (99.33 per cent),

Table 26. Effect of plant products on local lesion host in the control of CAMV (Pre-inoculation)

| Sl.No. | Treatments | Mean No. of lesions* | Inhibition over control (%) |
|---|---|---|---|
| 1. | Andrographis paniculata (Burm.f.) Wall | 16.4 | 72.67 |
| 2. | Azadirachta indica A.Juss. | 16.4 | 72.67 |
| 3. | Basella rubra L. | 0.2 | 99.67 |
| 4. | Boerhavia diffusa L. | 1.4 | 97.67 |
| 5. | Bougainvillea spectabilis Willd. | 0.4 | 99.33 |
| 6. | Capsicum annum L. | 0.0 | 100.00 |
| 7. | Casuarina equisetifolia L. | 6.2 | 89.67 |
| 8. | Catharanthus roseus (L.) G.Don. | 31.8 | 47.00 |
| 9. | Cocos nucifera L. | 11.2 | 81.33 |
| 10. | Croton bonplandianum Baill. | 0.2 | 99.67 |
| 11. | Curcuma longa L. | 38.0 | 36.67 |
| 12. | Datura stramonium L. | 0.8 | 98.67 |
| 13. | Gomphrena globosa L. | 9.2 | 84.67 |
| 14. | Ipomoea carnea Jacq. | 0.2 | 99.67 |
| 15. | Leucaena leucocephala (Lamk.) de Wit. | 4.2 | 93.00 |
| 16. | Mirabilis jalapa L. | 0.2 | 99.67 |
| 17. | Nerium indicum Mill. | 23.4 | 61.00 |
| 18. | Ocimum sanctum L. | 31.0 | 48.33 |
| 19. | Polyalthia longifolia Benth. & Hk.f. | 6.6 | 89.00 |
| 20. | Pongamia pinnata (L.) Pierre. | 2.2 | 96.33 |
| 21. | Prosopis chilensis (Molina.) Stuntz | 0.8 | 98.67 |
| 22. | Santalum album L. | 6.2 | 89.67 |
| 23. | Sorghum vulgare L. Pers. | 40.4 | 32.67 |
| 24. | Trianthema portulacastrum L. | 3.2 | 94.67 |
| 25. | Vitis nigundo L. | 11.2 | 81.33 |
| 26. | Control | 60.0 | - |
| | C.D. (P = 0.05) | 0.687 | |

* Mean of four replications

D.stramonium (98.67 per cent), P.chilensis (98.67 per cent), B.diffusa (97.67 per cent), P.pinnata (96.33 per cent), T.portulacastrum (94.67 per cent) and L.leucocephala (93.00 per cent).

4.8.2.2 Efficacy of plant extracts on combined inoculation

To detect the viral inhibitory activity on combined inoculation, equal amounts of plant extracts and virus inoculum were mixed and inoculated onto assay host (the controls consisted of virus inoculum mixed with equal volumes of distilled water).

The results in Table 27 indicated that there was significant inhibition of CAMV, following combined inoculation. Among the different combinations tried, four treatments (Vi + B.spectabilis, Vi + C.annum, Vi + C.bonplandianum and Vi + M.jalapa) showed cent per cent viral inhibitory activity over control followed by Vi + B.rubra, Vi + P.chilensis, Vi + B.diffusa, Vi + I.carnea, Vi + T.portulacastrum and Vi + N.indicum with per cent reduction in virus infection ranging from 92.77 to 98.8 over control.

Table 27. Effect of plant products on local lesion host in the control of CAMV on combined inoculation

| Sl. No. | Treatments | Mean No. of lesions* | Inhibition over control |
|---|---|---|---|
| 1. | Vi + *Andrographis paniculata* | 9.25 | 77.71 |
| 2. | Vi + *Azadirachta indica* | 7.50 | 81.93 |
| 3. | Vi + *Basella rubra* | 0.50 | 98.80 |
| 4. | Vi + *Boerhavia diffusa* | 1.50 | 96.39 |
| 5. | Vi + *Bougainvillea spectabilis* | 0.00 | 100.00 |
| 6. | Vi + *Capsicum annum* | 0.00 | 100.00 |
| 7. | Vi + *Casuarina equisetifolia* | 10.00 | 75.90 |
| 8. | Vi + *Catheranthus roseus* | 9.25 | 77.71 |
| 9. | Vi + *Cocos nucifera* | 5.25 | 87.35 |
| 10. | Vi + *Croton bonplandianum* | 0.00 | 100.00 |
| 11. | Vi + *Curcuma longa* | 19.50 | 53.01 |
| 12. | Vi + *Datura stramonium* | 8.00 | 80.72 |
| 13. | Vi + *Gomphrena globosa* | 10.50 | 74.70 |
| 14. | Vi + *Ipomoea carnea* | 1.50 | 96.39 |
| 15. | Vi + *Leucaena leucocephala* | 14.75 | 64.46 |
| 16. | Vi + *Mirabilis jalapa* | 0.00 | 100.00 |
| 17. | Vi + *Nerium indicum* | 3.00 | 92.77 |
| 18. | Vi + *Ocimum sanctum* | 7.00 | 83.13 |
| 19. | Vi + *Polyalthia longifolia* | 6.75 | 83.74 |
| 20. | Vi + *Pongamia pinnata* | 7.00 | 83.13 |
| 21. | Vi + *Prosopis chilensis* | 0.75 | 98.19 |
| 22. | Vi + *Santalum album* | 7.75 | 81.33 |
| 23. | Vi + *Sorghum vulgare* | 12.50 | 69.88 |
| 24. | Vi + *Trianthema portulacastrum* | 2.25 | 94.58 |
| 25. | Vi + *Vitis nigundo* | 20.00 | 51.81 |
| 26. | Control | 41.50 | |
| | CD (P = 0.05) | 0.617 | |

Vi = Virus inoculum; * Mean of four replications

4.8.2.3. Protein fraction from plant extracts, showing antiphytoviral properties

The observations in Table 28 indicated that addition of ammonium sulphate to clarified aqueous leaf extracts could precipitate the protein faction at different levels of saturation. Sixty per cent saturation of ammonium sulphate in *M.jalapa* leaf extracts could precipitate out 1504 µg out of 1585 µg $g^{-1}$ leaf sample. The same level of saturation (60%) also precipitated the protein at its maximum in *B.spectabilis* and *P.chilensis*, which were 1796 and 1848 µg respectively. In *I.carnea* 40 per cent saturation could precipitate the maximum of 1770 µg out of 1790 µg $g^{-1}$ in fresh leaf.

4.8.2.4. Effect of protein fractions on CAMV inhibition

The bioassay on the efficacy of different protein fractions in the inhibition of CAMV are presented in Table 29. The results showed that the protein fractions of the selected plant species were found to be highly effective in reducing the CAMV infection ranging from 30.2 to 100 per cent over control.

A maximum inhibition of 97.7 per cent was found in the protein fraction with 60 per cent ammonium sulphate saturation in *M.jalapa*, followed by 100, 40, 80 and 20 per cent when compared

Table 28. Protein fractionation from plant extracts showing antiviral properties

| Source of AVPs | ug g-1 fresh weight of tissue |
|---|---|
| a) *Mirabilis jalapa* | |
| i) Total protein | 1585 |
| ii) 20% saturation | 542 |
| iii) 40% saturation | 1159 |
| iv) 60% saturation | 1504 |
| v) 80% saturation | 1444 |
| vi) 100% saturation | 1493 |
| b) *Bougainvillea spectabilis* | |
| i) Total protein | 1815 |
| ii) 20% saturation | 783 |
| iii) 40% saturation | 1782 |
| iv) 60% saturation | 1796 |
| v) 80% saturation | 1785 |
| vi) 100% saturation | 1784 |
| c) *Ipomoea carnea* | |
| i) Total protein | 1791 |
| ii) 20% saturation | 1763 |
| iii) 40% saturation | 1770 |
| iv) 60% saturation | 1758 |
| v) 80% saturation | 1765 |
| vi) 100% saturation | 1716 |
| d) *Prosopis chilensis* | |
| i) Total protein | 1857 |
| ii) 20% saturation | 980 |
| iii) 40% saturation | 1798 |
| iv) 60% saturation | 1848 |
| v) 80% saturation | 1509 |
| vi) 100% saturation | 1631 |

Table 29. Effect of protein fractions on local lesion production by CAMV on *Chenopodium amaranticolor*

| Source of antiviral protein fractions | Mean No.of lesions (No.) | Inhibition (%) over control |
|---|---|---|
| a) *Mirabilis jalapa* | | |
| i) 20% | 12.0 | 30.2 |
| ii) 40% | 1.8 | 89.5 |
| iii) 60% | 0.4 | 97.7 |
| iv) 80% | 2.4 | 86.1 |
| v) 100% | 0.6 | 96.5 |
| vi) Control | 17.6 | |
| b) *Bougainvillea spectabilis* | | |
| i) 20% | 5.6 | 89.6 |
| ii) 40% | 3.0 | 94.4 |
| iii) 60% | 0.4 | 99.3 |
| iv) 80% | 0.0 | 100.0 |
| v) 100% | 0.0 | 100.0 |
| vi) Control | 53.6 | |
| c) *Ipomoea carnea* | | |
| i) 20% | 5.6 | 79.1 |
| ii) 40% | 3.0 | 88.8 |
| iii) 60% | 0.2 | 99.3 |
| iv) 80% | 0.0 | 100.0 |
| v) 100% | 0.0 | 100.0 |
| vi) Control | 26.8 | |
| d) *Prosopis chilensis* | | |
| i) 20% | 10.6 | 82.9 |
| ii) 40% | 6.0 | 90.3 |
| iii) 60% | 4.0 | 93.5 |
| iv) 80% | 0.6 | 99.0 |
| v) 100% | 0.0 | 100.0 |
| vi) Control | 61.8 | |

to control. Protein fractions from *B.spectabilis* showed more antiviral activity compared to other species tried. Cent per cent inhibition was found with 80 and 100 per cent saturation followed by 99.3, 94.4 and 89.6 with 60, 40 and 20 per cent respectively. Two fractions (80 and 100 per cent saturation) from *I.carnea* also showed cent per cent inhibition of CAMV followed by 99.33, 88.8 and 79.1 with 60, 40 and 20 per cent saturation. The protein fractions in *P.chilensis* also showed antiviral activity which recorded a maximum of 100 per cent with 100 per cent saturation followed by 99.0, 93.5, 90.3 and 82.9 with 80, 60, 40 and 20 per cent saturation respectively (Plates 36 to 39).

#### 4.8.2.5 Effect of partially purified Bougainvillea antiviral protein (BAP) in induction of defence mechanisms

##### a) Changes in peroxidase (PO) activity

The results (Table 30) indicated that the peroxidase activity was enhanced in cowpea seedlings following treatment with BAP at all periods of sampling in susceptible and resistant cultivar, when compared to untreated control. Treatment with BAP progressively increased the peroxidase activity and reached its maximum at 5 days after treatment in the susceptible varieties (29.02 and 23.91 per cent more than the control in CO 4 and C 152 respectively). However the increase was negligible in untreated healthy controls.

PLATE 36. Effect of *Mirabilis* antiviral protein on the infectivity of CAMV.

T1 - 20% protein fraction

T2 - 40% protein fraction

T3 - 60% protein fraction

T4 - 80% protein fraction

T5 - 100% protein fraction.

C - Control

PLATE 37. Effect of *Bougainvillea* antiviral protein on the infectivity of CAMV.

T1 - 20% protein fraction

T2 - 40% protein fraction

T3 - 60% protein fraction

T4 - 80% protein fraction

T5 - 100% protein fraction.

C - Control.

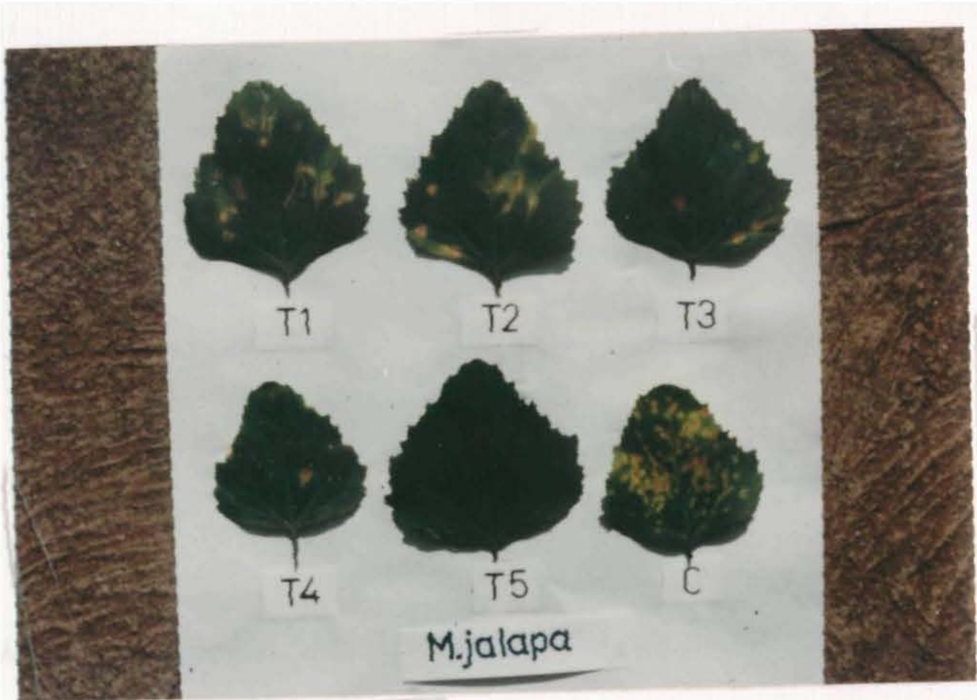



PLATE 36

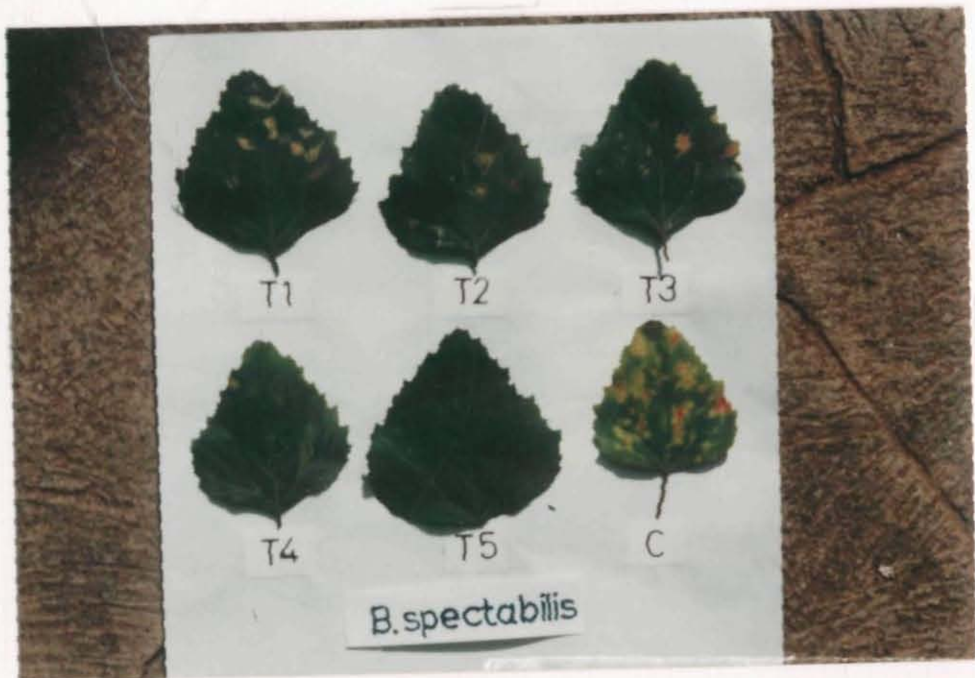



PLATE 37

PLATE 38. Effect of *Prosopis* antiviral protein on the infectivity of CAMV.

T1 - 20% protein fraction

T2 - 40% protein fraction

T3 - 60% protein fraction

T4 - 80% protein fraction

T5 - 100% protein fraction

C - Control.

PLATE 39. Effect of *Ipomoea* antiviral protein on the infectivity of CAMV.

T1 - 20% protein fraction

T2 - 40% protein fraction

T3 - 60% protein fraction

T4 - 80% protein fraction

T4 - 100% protein fraction

C - Control.

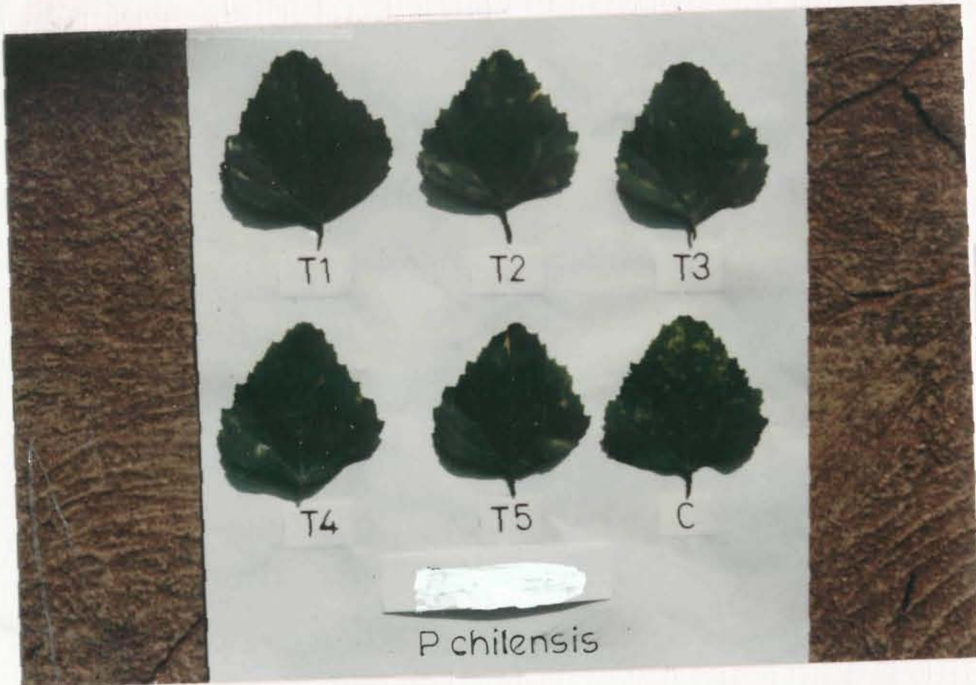



PLATE 38

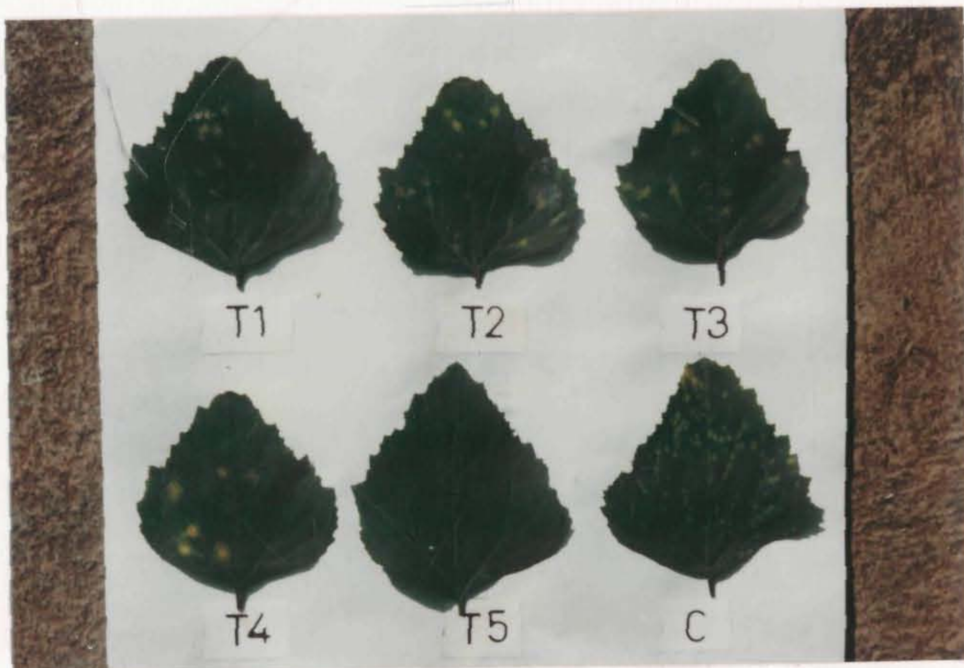



PLATE 39

Table 30. Changes in peroxidase activity in cowpea varieties sprayed with AVPs from *Bougainvillea spectabilis* (BAP)

| Treatments | | Peroxidase activity (Changes in absorbance $min^{-1}$ $g^{-1}$ fresh weight) at different days after treatment | | | |
|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 5 |
| **Susceptible varieties** | | | | | |
| 1. CO 4 | Sprayed | 38.200 (+10.41) | 39.900 (+17.35) | 44.400 (+29.07) | 44.900 (+29.02) |
| | Control | 34.600 | 34.000 | 34.400 | 34.800 |
| 2. C 152 | Sprayed | 32.000 (+ 3.90) | 34.300 (+13.95) | 35.800 (+12.58) | 39.900 (+23.91) |
| | Control | 30.800 | 30.100 | 31.800 | 32.200 |
| **Resistant variety** | | | | | |
| 1. CO 6 | Sprayed | 45.800 (+23.12) | 50.500 (+31.51) | 55.200 (+41.90) | 49.800 (+26.72) |
| | Control | 37.200 | 38.400 | 38.900 | 39.300 |
| CD (P=0.05) | | 4.200 | 2.723 | 2.696 | 2.041 |

Figures in parentheses represent per cent increase (+) over healthy (control)

The enzyme activity was very high in treated resistant variety, CO 6. The enzyme activity was at its peak at 3 days after spraying and showed a decline when the samples were analysed at 5 days after treatment, however the activity was more when compared to susceptible cultivars. There was a gradual increase in PO activity in untreated control plants (Fig. 14).

b) Changes in polyphenol oxidase (PPO) activity

The changes in PPO activity in cowpea seedlings following application of BAP are presented in Table 31. The results indicated that the PPO activity progressively increased with increase in plant age in susceptible and resistant plants.

Treatment of cowpea seedlings induced progressive increase in PPO activity and reached its maximum at 3 days after treatment and showed a decreased level at 5 days after treatment in both the susceptible varieties tested. Maximum activity of 47.22 and 27.08 per cent was observed in CO 4 and C 152 respectively.

The PPO activity also showed the same trend in the resistant cultivar. The peak enzyme activity of 36.21 per cent more than the control was observed in CO 6 variety at 3 days after treatment with BAP (Fig. 15).

**Fig.14 Changes in peroxidase activity in cowpea varieties sprayed with AVP from *Bougainvillea spectabilis*(BAP)**

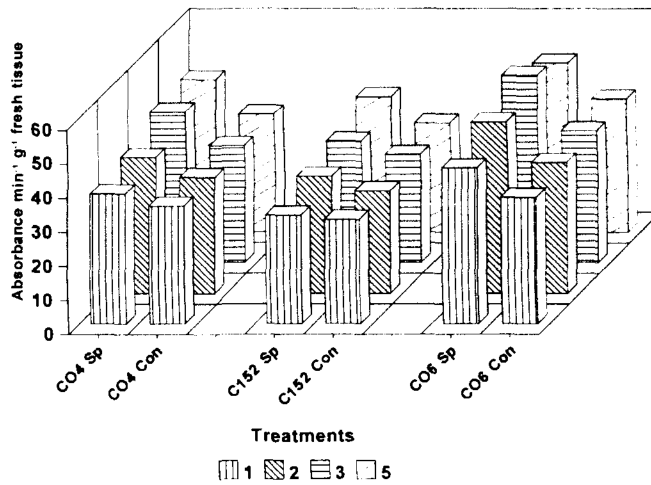
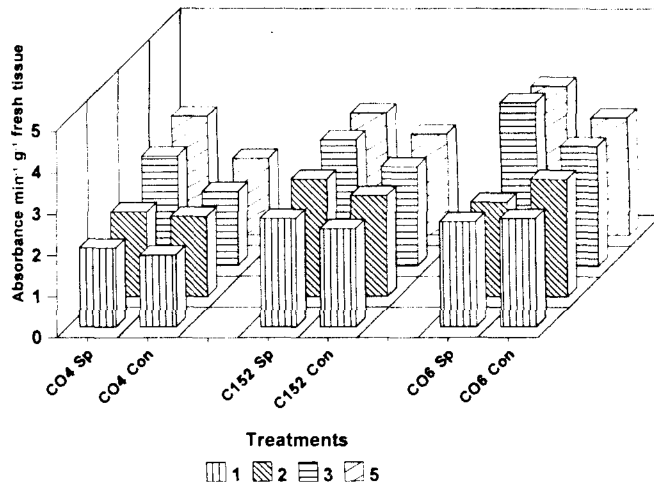


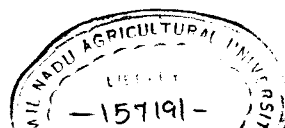
Table 31. Changes in polyphenol oxidase activity in cowpea varieties sprayed with AVPs from *Bougainvillea spectabilis* (BAP)

| Treatments | | Polyphenol oxidase activity (Changes in absorbance min-1 g-1 fresh weight) at different days after treatment | | | |
|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 5 |
| *Susceptible varieties* | | | | | |
| 1. CO 4 | Sprayed | 1.920 (+ 9.71) | 2.063 (+ 5.80) | 2.650 (+47.22) | 2.870 (+55.14) |
| | Control | 1.750 | 1.950 | 1.800 | 1.850 |
| 2. C 152 | Sprayed | 2.650 (+10.42) | 2.850 (+16.33) | 3.050 (+27.08) | 2.950 (+20.90) |
| | Control | 2.400 | 2.450 | 2.400 | 2.440 |
| *Resistant variety* | | | | | |
| 3. CO 6 | Sprayed | 2.570 (- 3.02) | 3.290 (+15.44) | 3.950 (+36.21) | 3.600 (+26.32) |
| | Control | 2.650 | 2.850 | 2.900 | 2.850 |
| CD (P=0.05) | | 0.182 | 0.230 | 0.446 | 0.207 |

Figures in parentheses represent per cent increase (+) or decrease (-) over healthy (control)

Fig.15 Changes in polyphenol oxidase activity in cowpea varieties sprayed with AVP from *Bougainvillea spectabilis*(BAP)

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c) Changes in phenylalanine ammonia-lyase (PAL) activity

The results of the experiments to determine the changes in PAL activity following treatment with BAP are presented in Table 32. The PAL activity showed an increasing trend which was proportional to the age of the plant in both susceptible and resistant cultivar analysed.

The susceptible varieties CO 4 and C 152 showed a consistent increase in PAL activity following treatment with BAP and reached maximum at 5 days after treatment. A maximum per cent increase of 34.26 and 46.86 was found in CO 4 and C 152 respectively.

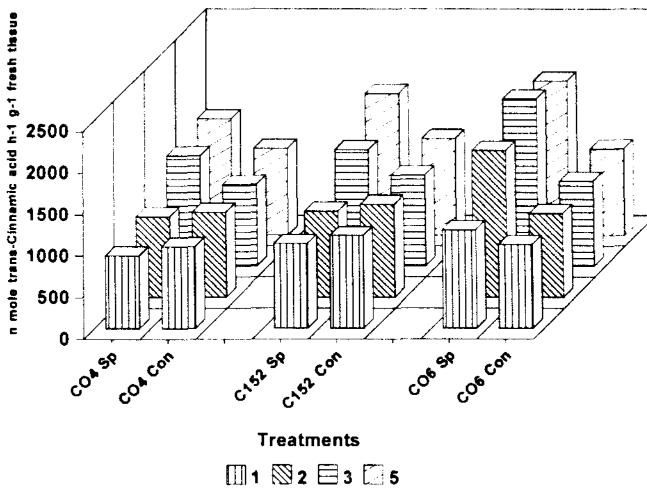
In resistant cultivar the PAL activity was induced much earlier when compared to susceptible varieties. The PAL activity was at its peak at 3rd day of sampling. The enzyme activity showed an increase of 96.22 per cent than that of control untreated plant. However, the activity showed a decreasing trend at 5 days after treatment but was higher than the susceptible variety compared in the experiment (Fig. 16).

Table 32. Changes in phenylalanine ammonia lyase activity in cowpea varieties sprayed with AVPs from *Bougainvillea spectabilis* (BAP)

| Treatments | | Changes in PAL activity (n moles trans-cinnamic acid $h^{-1}$ $g^{-1}$ fresh weight) at different days after treatment | | | |
|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 5 |
| **Susceptible varieties** | | | | | |
| 1. CO 4 | Sprayed | 872.000 (+11.20) | 979.000 (- 4.86) | 1335.000 (+35.40) | 1407.000 (+ 34.25) |
| | Control | 982.000 | 1029.000 | 986.000 | 1048.000 |
| 2. C 152 | Sprayed | 1021.000 (- 8.84) | 1042.000 (- 7.30) | 1409.000 (+27.86) | 1715.333 (+46.86) |
| | Control | 1120.333 | 1124.000 | 1102.000 | 1168.000 |
| **Resistant variety** | | | | | |
| 3. CO 6 | Sprayed | 1192.000 (+17.90) | 1776.000 (+74.46) | 2025.000 (+96.22) | 1871.000 (+78.87) |
| | Control | 1011.000 | 1018.000 | 1032.000 | 1046.000 |
| CD (P=0.05) | i) | 7.969 | 4.493 | 8.737 | 14.415 |
| | ii) | 4.601 | 2.594 | 5.044 | 8.322 |
| | iii) | 5.635 | 3.177 | 6.178 | 10.193 |

Figures in parentheses represent per cent increase (+) or decrease (-) over healthy (control)

**Fig.16 Changes in phenylalanine ammonia-lyase activity in cowpea varieties sprayed with AVP from *Bougainvillea spectabilis*(BAP)**



### 4.8.3 Management of CAMV disease under field condition

A field trial was carried out during December 1994 to find out the efficacy of selected botanicals and insecticides in the management of CAMV disease in cowpea under field conditions. The effect was assessed based on the incidence of CAMV disease, insect population and yield parameters of cowpea (Plates 40 and 41).

#### 4.8.3.1 Efficacy on the growth of the plants

The effect of different treatments mentioned in 3.11.3 were assessed in terms of height of the plants, length of roots and fresh and dry weights at 30 and 60 days after sowing.

The results (Table 33) showed that the CAMV infection has caused conspicuous reduction at 30 DAS in the height of plants in control compared to the treated plants. A maximum height of 26.7 cm was found in plants treated with monocrotophos (0.05%), which was 58.18 per cent more than the control, followed by MJLE, ICLE, BSLE, PCLE, mahua oil and neem oil. Leaf extracts performed better than the oil application. However there was not much difference between treatments. The length of roots also showed the same trend. A maximum mean length of 13.53 cm was observed in BSLE treated plants which was 48.88 per cent more

PLATE 40

PLATE 41

than the control. Fresh and dry weights also showed a significant increase in treated plants. Maximum mean fresh weight of 26.18 g was obtained in monocrotophos treated plants when compared to control (13.63 g). Plants treated with leaf extracts were superior in growth characters to the oil treated plants. A similar observation was found in dry weight of the plants also.

The growth parameters recorded in Table 34 at 60 DAs also showed a general reduction in height of plants and length of roots in the untreated control when compared with the treated. Plants treated with monocrotophos (0.05 per cent) showed a mean height of 106.33 cm and mean length of root, 17.323 cm which was superior among the treatments compared. Similar trend was observed in the case of fresh and dry weights which recorded 110.833 and 24.387 g respectively (Plates 42a to 42h).

#### 4.8.3.2 Efficacy on the incidence of CAMV disease

A marked inhibition of CAMV disease was observed in plants treated with leaf extracts and insecticide over control. The observations were recorded at 10, 30, 60 and 90 DAS and presented in Table 35.

A significant difference was observed in treated plants from 30 DAS. A maximum per cent inhibition of 86.82 was observed

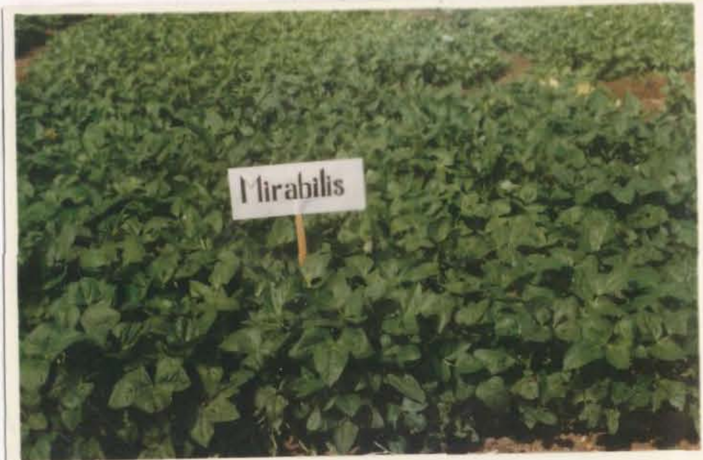
**Table 34.** Efficacy of botanicals and insecticides on the growth of cowpea plants
b) Sixty days after sowing

| Sl. No. | Treatments | Concen-tration (%) | Height of the plant | No. of leaves | Length of root | Fresh weight | Dry weight |
|---|---|---|---|---|---|---|---|
| 1. | BSLE | 10 | 84.447 | 14.557 | 16.377 | 61.667 | 16.870 |
| 2. | MJLE | 10 | 93.333 | 13.887 | 16.670 | 54.700 | 15.347 |
| 3. | ICLE | 10 | 95.443 | 16.443 | 17.177 | 74.333 | 17.673 |
| 4. | PCLE | 10 | 83.777 | 11.000 | 15.457 | 48.167 | 15.410 |
| 5. | Neem oil | 3 | 76.110 | 13.667 | 14.933 | 68.567 | 18.017 |
| 6. | Mahua oil | 3 | 69.333 | 12.333 | 15.287 | 68.667 | 17.090 |
| 7. | Monocrotophos | 0.05 | 106.330 | 15.890 | 17.323 | 110.833 | 24.387 |
| 8. | Control (Water) | | 46.557 | 9.777 | 14.510 | 48.667 | 13.400 |
| | C.D. (P=0.05) | | 14.752 | 2.758 | 2.629 | 16.987 | 3.557 |
| | C.D. (P=0.01) | | 20.474 | 3.828 | 3.649 | 23.576 | 4.937 |

PLATE 42

42 a

42 b




42 c

42d

42e

PLATE 42

42 a

42 f

42 g

42h

Table 35. Efficacy of botanicals and insecticide on the incidence of CAMV disease

| Sl. No. | Treatments | Concentration (%) | No. of plants infected out of 200 plants per plot | | | | Per cent inhibition over control | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Days after sowing | | | | Days after sowing | | | |
| | | | 10 | 30 | 60 | 90 | 10 | 30 | 60 | 90 |
| 1. | BSLE | 10 | 1.67 | 4.33 | 7.33 | 14.00 | 37.45 | 65.83 | 85.14 | 88.00 |
| 2. | MJLE | 10 | 1.00 | 3.00 | 8.33 | 13.67 | 62.55 | 76.32 | 83.11 | 88.28 |
| 3. | ICLE | 10 | 1.00 | 3.67 | 14.00 | 21.00 | 62.55 | 71.03 | 71.62 | 82.00 |
| 4. | PCLE | 10 | 2.67 | 5.67 | 11.33 | 20.67 | 0.00 | 55.25 | 77.03 | 82.28 |
| 5. | Neem oil | 3 | 0.33 | 3.00 | 5.00 | 9.33 | 87.64 | 76.32 | 89.86 | 92.00 |
| 6. | Mahua oil | 3 | 0.67 | 1.67 | 5.33 | 13.00 | 74.91 | 86.82 | 89.20 | 88.86 |
| 7. | Monocrotophos | 0.05 | 1.33 | 2.67 | 8.00 | 14.00 | 50.19 | 79.93 | 83.78 | 88.00 |
| 8. | Control (water) | | 2.6 | 12.67 | 49.33 | 116.67 | | | | |
| | CD (P=0.05) | | 0.51 | 0.87 | 1.17 | 1.23 | | | | |

in plants sprayed with mahua oil, followed by monocrotophos (79.93 per cent). MJLE and neem oil sprays gave an inhibition of 76.32 per cent which were on par. PCLE was inferior when compared with BSLE.

A maximum of 92 per cent inhibition was observed over control in neem oil sprayed plot. The treatments mahua oil, MJLE, BSLE and monocrotophos gave a mean per cent inhibition of 88.29, followed by PCLE and ICLE with an average inhibition of 82.14 per cent at 90 DAS (Fig. 17).

4.8.3.3 Efficacy on aphid population

The aphid population was assessed based on number of plants infected with the aphids out of 200 plants per treatment at 10, 30, 60 and 90 DAS. The mean number of plants infested with aphids were recorded at different periods are presented in Table 36.

The result indicated that there was a progressive increase in number of plants infected with aphids at different periods of sampling in untreated control plots. Maximum inhibition of cent per cent was observed at 30 and 60 DAS in monocrotophos (0.05 per cent) treated plots and was superior to other treatments tested. The oil sprays (Neem and Mahua oils)

**Fig.17 Efficacy of botanicals and insecticide on inhibition of CAMV disease**

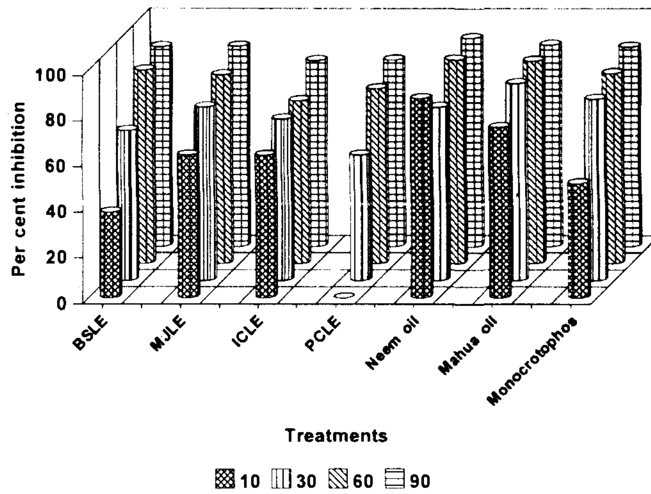


Table 36. Efficacy of botanicals and insecticide on the population of *Aphis craccivora*

| Sl. | Treatments | Number of plants with aphids per 200 plants | | | | Inhibition over control(%) | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 10 DAS | 30 DAS | 60 DAS | 90 DAS | 10 DAS | 30 DAS | 60 DAS | 80 DAS |
| 1. | BSLE | 10.67 | 52.33 | 80.67 | 41.33 | 38.43 | 53.15 | 48.84 | 58.81 |
| 2. | MJLE | 6.67 | 38.33 | 58.00 | 28.67 | 61.51 | 65.47 | 63.21 | 71.42 |
| 3. | ICLE | 5.33 | 35.67 | 68.00 | 51.67 | 69.24 | 67.86 | 56.87 | 48.50 |
| 4. | PCLE | 9.67 | 60.33 | 71.33 | 44.00 | 44.20 | 45.65 | 54.76 | 56.14 |
| 5. | Neem oil | 4.00 | 7.00 | 10.00 | 11.00 | 76.92 | 93.69 | 93.66 | 89.04 |
| 6. | Mahua oil | 4.00 | 8.33 | 13.00 | 8.00 | 76.92 | 92.50 | 91.75 | 92.03 |
| 7. | Monocrotophos | 3.67 | 0.00 | 0.00 | 4.67 | 78.82 | 100.00 | 100.00 | 95.34 |
| 8. | Control | 17.33 | 111.00 | 157.67 | 100.33 | - | - | - | - |
| | CD (P=0.05) | 0.65 | 2.84 | 1.02 | 2.09 | | | | |

were also found to be superior to plant extracts in reducing the aphid population. Among the plant extracts tested, MJLE gave maximum reduction of aphid population (Fig. 18).

4.8.3.4 Efficacy on the yield of cowpea

The results (Table 37) indicated that there was marked reduction in number of grains per pod, hundred grain weight and yield per ha in untreated control plots. A significant variation in grain yield was observed among different treatments. The maximum yield of 872.5 kg ha-1 could be obtained from BSLE treated plots followed by monocrotophos and MJLE where the yields recorded 833.3 and 830.8 kg h-1 respectively. The lowest (455.8 kg ha-1) was recorded in control (Fig. 19).

4.8.4 Effect of plant extracts and insecticide on insect transmission and survival (Glasshouse)

4.8.4.1 Efficacy on the transmission of CAMV by aphids

The efficiency of four plant extracts and one insecticide were evaluated and the data are presented in Table 38.

The results showed that the incubation period of the virus was found to increase in all treatments with plant extracts. Incubation period in plants treated with MJLE recorded the maximum (20 days) followed by BSLE, ICLE and PCLE which recorded 17.5, 17.5 and 17.0 days against 13.5 days in control.

Fig.18 Efficacy of botanicals and insecticide on the population of *Aphis craccivora*

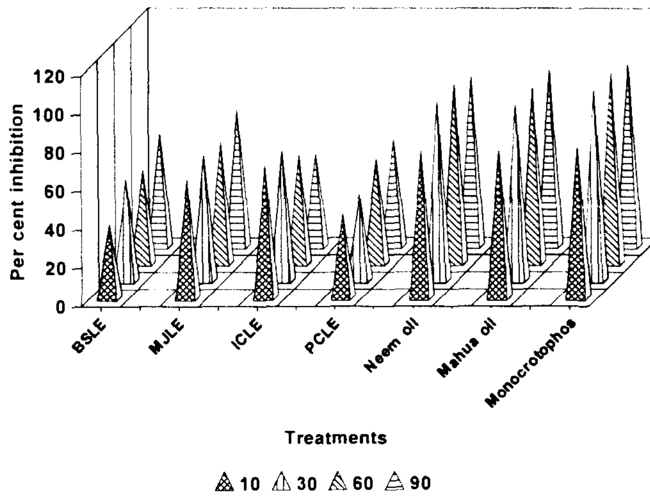


Table 37. Efficacy of botanicals and insecticides on the yield of cowpea

| Sl. No. | Treatments | No. of grains per pod | Hundred grain weight | Total grain yield in $12m^2$ | Yield (Kg/ha) | Per cent increase over control (Yield) |
|---|---|---|---|---|---|---|
| 1. | BSLE | 16.667 | 11.240 | 1.047 | 872.5 | 91.42 |
| 2. | MJLE | 16.833 | 10.993 | 0.997 | 830.8 | 82.27 |
| 3. | ICLE | 15.500 | 10.613 | 0.867 | 722.5 | 58.51 |
| 4. | PJLE | 15.083 | 10.523 | 0.813 | 677.5 | 48.64 |
| 5. | Neem oil | 15.667 | 10.827 | 0.740 | 616.7 | 35.30 |
| 6. | Mahua oil | 17.083 | 10.870 | 0.673 | 560.8 | 23.04 |
| 7. | Monocrotophos | 17.750 | 11.387 | 1.000 | 833.3 | 82.82 |
| 8. | Control | 11.333 | 9.560 | 0.547 | 455.8 | - |
| | CD (P=0.01) | 2.591 | 0.773 | 0.007 | | |
| | CD (P=0.05) | 1.867 | 0.557 | 0.005 | | |

Fig.19 Efficacy of botanicals and insecticide on the yield of cowpea

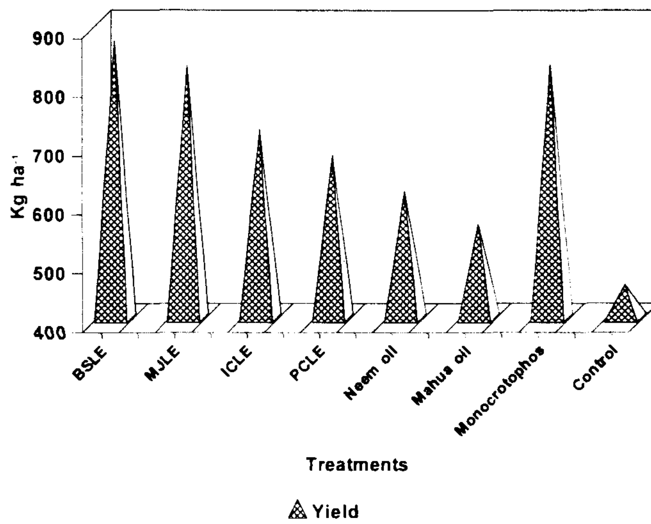


Table 38. Effect of plant extracts and insecticide on transmission of CAMV (Glasshouse condition)

| Treatments | Plants infected/ plants exposed# (No.) | Incubation period (days) | Plants infected (%) | Inhibition over control |
|---|---|---|---|---|
| BSLE | 4/20 | 17.5 | 20 | 75.0 |
| MJLE | 2/20 | 20.0 | 10 | 87.5 |
| ICLE | 6/20 | 17.5 | 30 | 62.5 |
| PCLE | 10/20 | 17.0 | 50 | 37.5 |
| Monocrotophos | 0/20 | - | 0 | 100.0 |
| Control (Water) | 16/20 | 13.5 | 80 | - |

* 10% plant extract and 0.05% monocrotophos was sprayed 24 h prior to inoculation access period at cotyledonary leaf stage (7 and 14 DAS)

# Groups of 5 viruliferous adult aphids were released on each cowpea seedlings

The per cent inhibition was cent per cent when sprayed with monocrotophos followed by MJLE, BSLE, ICLE and PCLE with a per cent reduction of 87.5, 75.0, 62.5 and 37.5 respectively.

4.8.4.2 Efficacy on the survival of aphids

All the treatments were found to be effective in the mortality of aphids (Table 39). There was cent per cent mortality when plants were treated with monocrotophos. Among the plant extracts evaluated the per cent survival was 10 with MJLE followed by BSLE (14%), ICLE (22%) and PCLE (27%) against 75 per cent in control. There was a marked difference in percentage of plants infected. None of the plants were infected on treatment with monocrotophos. The per cent infection in plants treated with MJLE, BSLE, ICLE and PCLE were 10, 20, 30 and 50 respectively against 80 per cent in control plants.

## 4.9 SCREENING FOR SOURCES OF RESISTANCE TO CAMV IN COWPEA GERMPLASM

Three hundred and thirty two genotypes were raised in glasshouse and inoculated artificially by mechanical inoculation. the observations on the incidence of CAMV disease was recorded and the results are presented in Table 40 showing per cent disease incidence and their reaction.

Table 39. Effect of plant extracts and insecticide on insect survival

| Treatments* | Per cent survival# | Plants infected/ plants exposed@ | Plants infected (%) |
|---|---|---|---|
| BSLE | 14 | 4/20 | 20 |
| MJLE | 10 | 2/20 | 10 |
| ICLE | 22 | 6/20 | 30 |
| PCLE | 27 | 10/20 | 50 |
| Monocrotophos | 0 | 0/20 | 0 |
| Control | 75 | 16/20 | 80 |

* 10% plant extract and 0.05% monocrotophos was sprayed 24 h prior to inoculation access period at cotyledonary leaf stage and 14 DAS

\# Number of released insects were counted 20 days after release

@ Groups of 5 viruliferous adults were released on each cowpea seedling

Table 40. Screening genotypes of cowpea (Glasshouse condition)

| Sl. No. | Genotypes screened | Disease incidence (%) | Reaction |
|---|---|---|---|
| 1. | CO 4 | 100.00 | HS |
| 2. | C 152 | 100.00 | HS |
| 3. | CO 6 | 0 | R |
| 4. | Kanakamony | 82.50 | HS |
| 5. | Pusakomal | 67.50 | HS |
| 6. | Pusa Dophasli | 60.00 | S |
| 7. | EC 240715 NBPGR | 90.00 | HS |
| 8. | Sel.263 (Ludhiana) | 88.23 | HS |
| 9. | Sel. 2-1 (Faizabad) | 46.15 | S |
| 10. | Arka garima | 100.00 | HS |
| 11. | Malika | 59.23 | S |
| 12. | Sharika | 66.67 | HS |
| 13. | Type - 3 | 20.00 | T |
| 14. | Type - 9 | 30.00 | T |
| 15. | Type - 17 | 14.20 | T |
| 16. | Type - 19 | 21.40 | T |
| 17. | Type - 19-1 | 100.00 | HS |
| 18. | Type - 20 | 83.30 | HS |
| 19. | Type - 21 | 20.00 | T |
| 20. | Type - 21-1 | 100.00 | HS |
| 21. | Type - 22 | 42.85 | S |
| 22. | Type - 22-1 | 34.78 | S |
| 23. | Type - 27 | 77.80 | HS |
| 24. | Type - 31 | 71.43 | HS |
| 25. | Type - 31-1 | 42.86 | S |
| 26. | Type - 32 | 47.06 | S |
| 27. | Type - 33 | 52.63 | S |
| 28. | Type - 34 | 100.00 | HS |
| 29. | Type - 36 | 80.00 | HS |
| 30. | Type - 36-1 | 33.00 | S |
| 31. | Type - 38 | 10.53 | T |
| 32. | Type - 38-1 | 20.69 | T |
| 33. | Type - 39 | 18.75 | T |
| 34. | Type - 40 | 30.00 | T |
| 35. | Type - 41 | 14.29 | T |
| 36. | Type - 41-1 | 14.29 | T |
| 37. | Type - 42 | 11.11 | T |
| 38. | Type - 43 | 66.67 | HS |
| 39. | Type - 44 | 10.00 | R |
| 40. | Type - 44-1 | 10.00 | R |
| 41. | Type - 45 | 10.50 | T |
| 42. | Type - 47 | 16.67 | T |

| Sl. No. | Genotypes screened | Disease incidence (%) | Reaction |
|---|---|---|---|
| 43. | Type - 49 | 20.00 | T |
| 44. | Type - 50 | 14.29 | T |
| 45. | Type - 50-1 | 12.00 | T |
| 46. | Type - 51 | 33.33 | S |
| 47. | Type - 52 | 16.67 | T |
| 48. | Type - 53 | 22.22 | T |
| 49. | Type - 53-1 | 10.53 | T |
| 50. | Type - 54 | 30.00 | T |
| 51. | Type - 55 | 66.67 | HS |
| 52. | Type - 56 | 35.00 | S |
| 53. | Type - 56-1 | 40.00 | S |
| 54. | Type - 57 | 50.00 | S |
| 55. | Type - 58 | 45.45 | S |
| 56. | Type - 59 | 54.55 | S |
| 57. | Type - 60 | 23.07 | T |
| 58. | Type - 61 | 30.76 | S |
| 59. | Type - 62 | 60.00 | S |
| 60. | Type - 63 | 40.00 | S |
| 61. | Type - 64 | 50.00 | S |
| 62. | Type - 64-1 | 100.00 | HS |
| 63. | Type - 64-2 | 100.00 | HS |
| 64. | Type - 65 | 14.28 | T |
| 65. | Type - 65-1 | 18.75 | T |
| 66. | Type - 66-0 | 66.67 | HS |
| 67. | Type - 66-1 | 20.00 | T |
| 68. | Type - 67 | 75.00 | HS |
| 69. | Type - 67-1 | 33.33 | S |
| 70. | Type - 68 | 100.00 | HS |
| 71. | Type - 69 | 21.43 | T |
| 72. | Type - 70 | 18.75 | T |
| 73. | Type - 71 | 36.36 | S |
| 74. | Type - 72 | 10.00 | R |
| 75. | Type - 73 | 36.36 | S |
| 76. | Type - 73-1 | 60.00 | S |
| 77. | Type 74 | 46.15 | S |
| 78. | Type - 74-2 | 31.25 | S |
| 79. | Type - 75 | 29.41 | T |
| 80. | Type - 76 | 25.00 | T |
| 81. | Type - 77 | 57.14 | S |
| 82. | Type - 78 | 100.00 | HS |
| 83. | Type - 79 | 28.00 | T |
| 84. | Type - 80 | 100.00 | HS |
| 85. | Type - 81 | 15.00 | T |
| 86. | Type - 81-1 | 66.67 | HS |
| 87. | Type - 82 | 40.00 | S |

| Sl. No. | Genotypes screened | Disease incidence (%) | Reaction |
|---|---|---|---|
| 88. | Type - 85 | 40.00 | S |
| 89. | Type - 85-1 | 50.00 | S |
| 90. | Type - 86 | 66.67 | HS |
| 91. | Type - 87 | 21.05 | T |
| 92. | Type - 87-1 | 30.00 | T |
| 93. | Type - 87-2 | 57.14 | S |
| 94. | Type - 88 | 30.00 | T |
| 95. | Type - 88-1 | 28.57 | T |
| 96. | Type - 89 | 9.09 | R |
| 97. | Type - 91 | 4.71 | R |
| 98. | Type - 92 | 30.77 | S |
| 99. | Type - 93 | 100.00 | HS |
| 100. | Type - 94 | 100.00 | HS |
| 101. | Type - 96 | 100.00 | HS |
| 102. | Type - 97 | 35.29 | S |
| 103. | Type - 98 | 75.00 | HS |
| 104. | Type - 99 | 50.00 | S |
| 105. | Type - 99-1 | 33.33 | S |
| 106. | Type -100 | 50.00 | S |
| 107. | Type -101 | 6.67 | R |
| 108. | Type -101-1 | 75.00 | HS |
| 109. | Type -101-2 | 10.00 | R |
| 110. | Type -102 | 57.14 | S |
| 111. | Type - 103 | 100.00 | HS |
| 112. | CGD - 198 | 50.00 | S |
| 113. | CGD - 199 | 5.00 | R |
| 114. | CGD - 199-1 | 4.55 | R |
| 115. | CGD - 200 | 16.67 | T |
| 116. | CGD - 201 | 16.67 | T |
| 117. | CGD - 202 | 50.00 | S |
| 118. | CGD - 203 | 50.00 | S |
| 119. | CGD - 204 | 87.50 | HS |
| 120. | CGD - 205 | 50.00 | S |
| 121. | CGD - 206 | 33.33 | S |
| 122. | CGD - 207 | 28.57 | T |
| 123. | CGD - 208 | 100.00 | HS |
| 124. | CGD - 209 | 11.11 | T |
| 125. | CGD - 210 | 11.43 | T |
| 126. | CGD - 211 | 4.65 | R |
| 127. | CGD - 213 | 28.57 | T |
| 128. | CGD - 214 | 6.67 | R |
| 129. | CGD - 218 | 50.00 | S |
| 130. | CGD - 220 | 16.67 | T |

| Sl. No. | Genotypes screened | Disease incidence (%) | Reaction |
|---|---|---|---|
| 131. | CGD - 223 | 28.57 | T |
| 132. | CGD - 225 | 7.69 | R |
| 133. | CGD - 226 | 66.67 | HS |
| 134. | CGD - 227 | 87.50 | HS |
| 135. | CGD - 229 | 80.00 | HS |
| 136. | CGD - 233 | 5.55 | R |
| 137. | CGD - 236 | 6.25 | R |
| 138. | CGD - 238 | 3.57 | R |
| 139. | CGD - 239 | 57.14 | S |
| 140. | CGD - 240 | 60.00 | S |
| 141. | CGD - 241 | 33.33 | S |
| 142. | CGD - 245 | 60.00 | S |
| 143. | CGD - 246 | 50.00 | S |
| 144. | CGD - 247 | 66.67 | HS |
| 145. | CGD - 256 | 90.32 | HS |
| 146. | P - 1461 | 70.00 | HS |
| 147. | P - 1473 | 50.00 | S |
| 148. | P - 1173-1 | 71.43 | HS |
| 149. | P - 1173-2 | 75.00 | HS |
| 150. | P - 1174 | 31.25 | S |
| 151. | PLS - 25 | 65.00 | HS |
| 152. | PLS - 38 | 20.00 | T |
| 153. | PLS - 38-1 | 71.43 | HS |
| 154. | PLS - 45 | 50.00 | S |
| 155. | PLS - 102 | 90.90 | HS |
| 156. | PLS - 110 | 68.18 | HS |
| 157. | PLS - 139 | 69.56 | HS |
| 158. | PLS - 232 | 93.75 | HS |
| 159. | MS - 1948 | 11.90 | T |
| 160. | MS - 1168 | 40.00 | S |
| 161. | MS - 8970 | 38.89 | S |
| 162. | MS - 8974 | 50.00 | S |
| 163. | MS - 8977 | 81.25 | HS |
| 164. | MS - 8978 | 7.50 | R |
| 165. | MS - 8989 | 91.67 | HS |
| 166. | MS - 1020 | 27.78 | T |
| 167. | MS - 1021 | 57.14 | S |
| 168. | MS - 9021-1 | 50.00 | S |
| 169. | MS - 9082 | 72.72 | HS |
| 170. | MS - 9080 | 80.00 | HS |
| 171. | MS - 9304 | 37.50 | S |
| 172. | MS - 9314 | 88.89 | HS |
| 173. | MS - 9369 | 22.22 | T |
| 174. | MS - 9558 | 47.00 | S |
| 175. | MS - 9558-1 | 83.33 | HS |

| Sl. No. | Genotypes screened | Disease incidence (%) | Reaction |
|---|---|---|---|
| 176. | MS - 9561 | 23.81 | T |
| 177. | MS - 9795 | 75.00 | HS |
| 178. | MS - 9796 | 28.57 | T |
| 179. | MS - 9805 | 13.33 | T |
| 180. | C - 20 | 20.00 | T |
| 181. | C - 57 | 6.25 | R |
| 182. | C - 23 | 75.00 | HS |
| 183. | C - 2099 | 75.00 | HS |
| 184. | C - 2779 | 6.25 | R |
| 185. | C - 8399 | 6.25 | R |
| 186. | V - 2 | 20.00 | T |
| 187. | V - 4 | 10.00 | R |
| 188. | V - 8 | 85.71 | HS |
| 189. | V - 12 | 5.55 | R |
| 190. | V - 14 | 27.27 | T |
| 191. | V - 16 | 20.00 | T |
| 192. | V - 18 | 9.52 | R |
| 193. | V - 23 | 13.33 | T |
| 194. | V - 26 | 5.00 | R |
| 195. | No - 1 | 40.00 | S |
| 196. | No - 58 | 60.00 | S |
| 197. | No - 58-1 | 40.00 | S |
| 198. | No - 96 | 10.00 | R |
| 199. | No - 96-1 | 50.00 | S |
| 200. | No - 99 | 6.67 | R |
| 201. | T - 495 | 71.43 | HS |
| 202. | T - 500 | 5.00 | R |
| 203. | T - 560 | 24.00 | T |
| 204. | T - 1148 | 20.00 | T |
| 205. | M - 1 | 75.00 | HS |
| 206. | M - 6/1 | 42.86 | S |
| 207. | M - 6/7 | 16.67 | T |
| 208. | M - 18 | 23.53 | T |
| 209. | CG - 11 | 85.71 | HS |
| 210. | CG - 28 | 50.00 | S |
| 211. | CG - 69 | 28.57 | T |
| 212. | EC - 42/6 | 6.25 | R |
| 213. | EC - 2045 | 13.33 | T |
| 214. | EC - 2857 | 6.25 | R |
| 215. | EC - 2858 | 6.25 | R |
| 216. | VITA - 3 | 12.50 | T |
| 217. | CTM - 4 | 33.33 | S |
| 218. | JA - 5 | 6.25 | R |
| 219. | G - 5 | 40.00 | S |
| 220. | G - 7 | 33.33 | S |

| Sl. No. | Genotypes screened | Disease incidence (%) | Reaction |
|---|---|---|---|
| 221. | CVU - 7 | 33.33 | S |
| 222. | RC - 8 | 30.77 | S |
| 223. | HM - III | 50.00 | S |
| 224. | K - 39 | 25.00 | T |
| 225. | CPS - 118 | 29.41 | T |
| 226. | ECR - 78-B | 13.33 | T |
| 227. | GCDE - 400 | 77.78 | HS |
| 228. | ECR - 1094 | 55.76 | S |
| 229. | GCDE - 402 | 9.09 | R |
| 230. | ECR - 1095 | 40.00 | S |
| 231. | Huspet | 37.50 | S |
| 232. | Iyyanpalayam | 6.67 | R |
| 233. | Kapour (Local) | 100.00 | HS |
| 234. | Puliampatty | 20.00 | T |
| 235. | Achipatty | 16.67 | T |
| 236. | Havanery | 6.67 | R |
| 237. | Vananthody | 100.00 | HS |
| 238. | Nagarkoil | 20.00 | T |
| 239. | Killiyor (B) | 10.00 | R |
| 240. | Killiyor (BR) | 60.00 | S |
| 241. | Pon-L-Block | 10.00 | R |
| 242. | Pollachi (North) | 47.37 | S |
| 243. | Tiruchi (Local) | 37.50 | S |
| 244. | Singapore | 50.00 | S |
| 245. | Type No. 100-1 | 33.33 | S |
| 246. | Type No. 138 | 100.00 | HS |
| 247. | Type No. 139 | 33.33 | S |
| 248. | Type No. 140 | 16.67 | T |
| 249. | Type No. 145 | 14.28 | T |
| 250. | Type No. 148-1 | 57.14 | S |
| 251. | Type No. 151 | 10.00 | R |
| 252. | Type No. 569 | 5.56 | R |
| 253. | Type No. 692-2 | 46.67 | S |
| 254. | Type No. 899 | 36.36 | S |
| 255. | Type No. 969 | 23.08 | T |
| 256. | NBPGR - 2 | 71.43 | HS |
| 257. | NBPGR - 5 | 60.00 | S |
| 258. | NBPGR - 7 | 62.07 | HS |
| 259. | NBPGR - 13 | 75.00 | HS |
| 260. | NBPGR - 14 | 82.16 | HS |
| 261. | NBPGR - 16 A | 57.14 | S |
| 262. | NBPGR - 17 A | 13.04 | T |
| 263. | NBPGR - 18 B | 30.77 | S |
| 264. | NBPGR - 24 | 100.00 | HS |
| 265. | NBPGR - 25 | 18.18 | T |

| Sl. No. | Genotypes screened | Disease incidence (%) | Reaction |
|---|---|---|---|
| 266. | NBPGR - 32 A | 85.71 | HS |
| 267. | NBPGR - 34 A | 30.00 | T |
| 268. | NBPGR - 52 | 87.50 | HS |
| 269. | NBPGR - 75 | 85.71 | HS |
| 270. | NBPGR - 125 A | 66.67 | HS |
| 271. | NBPGR - 129 | 70.00 | HS |
| 272. | NBPHR - 144 A | 100.00 | HS |
| 273. | NBPGR - 148 | 83.33 | HS |
| 274. | NBPGR - 158 | 52.63 | S |
| 275. | NBPGR - 164 | 61.54 | HS |
| 276. | NBPGR - 166 | 100.00 | HS |
| 277. | NBPGR - 170 | 90.00 | HS |
| 278. | NBPGR - 171 | 100.00 | HS |
| 279. | NBPGR - 176 | 100.00 | HS |
| 280. | NBPGR - 178 | 50.00 | S |
| 281. | NBPGR - 180-A | 100.00 | HS |
| 282. | NBPGR - 181 | 66.67 | HS |
| 283. | NBPHR - 188 | 66.67 | HS |
| 284. | NBPGR - 198 | 55.56 | S |
| 285. | NBPGR - 217 | 75.00 | HS |
| 286. | NBPGR - 218 | 75.00 | HS |
| 287. | NBPGR - 220-A | 76.92 | HS |
| 288. | NBPGR - 221 | 100.00 | HS |
| 289. | NBPGR - 228 | 62.86 | HS |
| 290. | NBPGR - 230 | 88.00 | HS |
| 291. | NBPGR - 233-A | 79.17 | HS |
| 292. | NBPGR - 236-A | 100.00 | HS |
| 293. | NBPGR - 244 | 100.00 | HS |
| 294. | NBPGR - 246-A | 46.15 | S |
| 295. | NBPGR - 248 | 66.67 | HS |
| 296. | NBPGR - 253 | 60.00 | S |
| 297. | NBPGR - 258 | 70.83 | HS |
| 298. | NBPGR - 282 | 57.69 | S |
| 299. | NBPGR - 285-A | 78.26 | HS |
| 300. | NBPGR - 317-A | 71.43 | HS |
| 301. | NBPGR - 319-A | 92.31 | HS |
| 302. | NBPGR - 403 | 100.00 | HS |
| 303. | NBPGR - 677 | 68.75 | HS |
| 304. | NBPGR - 744-A | 72.73 | HS |
| 305. | NBPGR - 748 | 66.67 | HS |
| 306. | NBPGR - 759 | 13.33 | T |
| 307. | NBPGR - 770 | 87.50 | HS |
| 308. | NBPGR - 783 | 100.00 | HS |
| 309. | NBPGR - 784 | 26.32 | T |
| 310. | NBPGR - 831 | 88.24 | HS |

| Sl. No. | Genotypes screened | Disease incidence (%) | Reaction |
|---|---|---|---|
| 311. | NBPGR - 976-A | 100.00 | HS |
| 312. | NBPGR - 998 | 100.00 | HS |
| 313. | NBPGR - 1084 | 80.00 | HS |
| 314. | NBPGr - 1095 | 83.33 | HS |
| 315. | NBPGR - 1107 | 100.00 | HS |
| 316. | NBPGR - 1153 | 100.00 | HS |
| 317. | NBPGR - 1164 | 25.00 | T |
| 318. | NBPGR - 1183 | 5.00 | R |
| 319. | NBPGR - 1195-B | 0 | R |
| 320. | NBPGR - 1442 | 80.00 | HS |
| 321. | NBPGR - 1501 | 68.42 | HS |
| 322. | NBPGR - 1508 | 100.00 | HS |
| 323. | NBPGR - 1518 | 100.00 | HS |
| 324. | NBPGR - 1547 | 100.00 | HS |
| 325. | NBPGR - 1562 | 88.24 | HS |
| 326. | NBPGR - 1563 | 100.00 | HS |
| 327. | NBPGR - 1581 | 100.00 | HS |
| 328. | NBPGR - 1593 | 100.00 | HS |
| 329. | NBPGR - 1599 | 25.00 | T |
| 330. | NBPGR - 1614 | 100.00 | HS |
| 331. | NBPGR - 1615 | 45.16 | S |
| 332. | NBPGR - 1621 | 82.35 | HS |

R = Resistant; T = Tolerant; S = Susceptible;
HS = Highly susceptible

| Per cent infection | Reaction |
|---|---|
| 0 - 10 | Resistant |
| 11 - 30 | Tolerant |
| 31 - 60 | Susceptible |
| 61 - 100 | Highly susceptible |

Among the germplasm entries screened, 40 were resistant to CAMV. Seventy eight showed tolerant reaction, 90 were susceptible and the remaining 124 entries were highly susceptible.

## 4.10 IN VITRO CALLUS CULTURE AND REGENERATION OF COWPEA

### 4.10.1 Callus induction

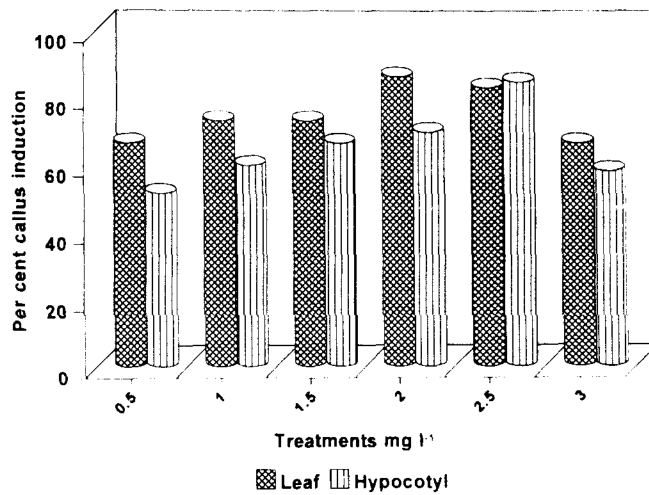
Leaf and hypocotyl explants from in vitro raised seedlings were used for callus induction. The basal medium MS supplemented with 6 levels of 2,4-D were used to select a suitable medium for callus induction. The response of the explants in different combinations of 2,4-D and a single level of kinetin are shown in Table 41 and Fig. 20.

The callus was induced in all the treatments. There was significant difference between treatments. The callus initiation occurred on 7th day in leaf explant and on 15th day in hypocotyl explant. Maximum callus induction per cent (86.67) was observed in MS + 2,4-D (2.0 mg $l^{-1}$) + Kn (1.0 mg $l^{-1}$) with leaf explant. The hypocotyl explant showed maximum callusing (85. per cent) in MS + 2,4-D (2.5 mg $l^{-1}$) + Kn (1.0 mg $l^{-1}$). The callus induction frequency ranged from 66.67 to 86.67 per cent in leaf explant and 51.67 to 85.00 per cent in hypocotyl explant.

Table 41. Effect of 2,4-D on callus induction frequency of leaf and hypocotyl explants

| Sl. No. | Treatments 2,4-D mg $l^{-1}$ | No.of explants callusing/explants inoculated | | Callus induction percentage | |
|---|---|---|---|---|---|
| | | Leaf | Hypocotyl | Leaf | Hypocotyl |
| 1. | 0.5 | 20/30 | 31/60 | 66.67 | 51.67 |
| 2. | 1.0 | 22/30 | 36/60 | 73.33 | 60.00 |
| 3. | 1.5 | 22/30 | 40/60 | 73.33 | 66.67 |
| 4. | 2.0 | 26/30 | 42/60 | 86.67 | 70.00 |
| 5. | 2.5 | 25/30 | 51/60 | 83.33 | 85.00 |
| 6. | 3.0 | 20/30 | 35/60 | 66.67 | 58.33 |

**Fig.20 Effect of 2, 4-D on callus induction frequency of leaf and hypocotyl explants**



The callus proliferation continued upto 30 days (Plates 43 to 45) in the callus induction medium. Calli formed were highly granular and creamy white in colour.

4.10.2 Callus regeneration

The response of calli obtained from leaf and hypocotyl explants tested on basal MS media fortified with six levels of BAP and NAA are presented in Table 42. The calli turned granular and proliferated again when subcultured into regeneration medium. After 30 days, further growth was inhibited and they turned brown in colour. There was no regeneration in any of the medium tried.

PLATE 43. Callus induction from hypocotyl explant (CO6, CO4, C152).

PLATE 44. Callus induction from leaf explant (CO4, C152)

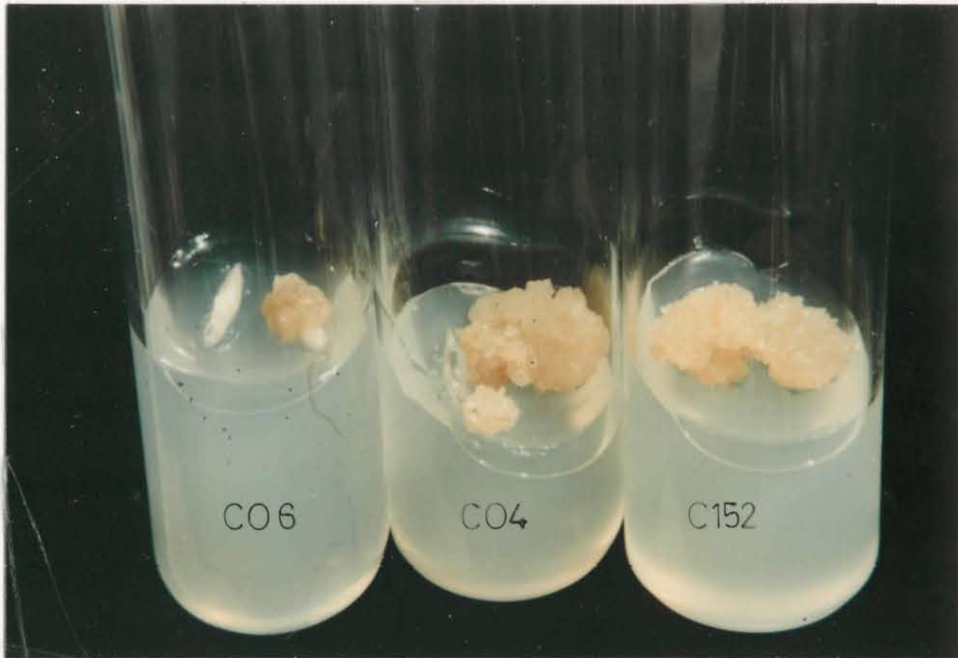



PLATE 43

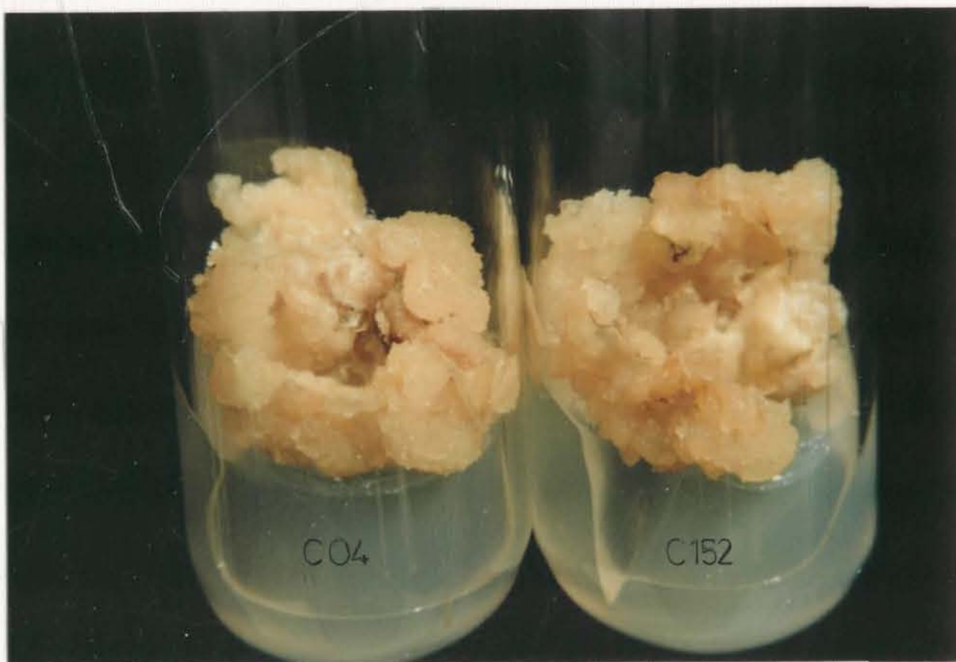



PLATE 44

PLATE 45. Callus induction from leaf explant showing rhizogenesis (CO6).

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PLATE 45

R- rhizogenesis

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Table 42. Response of calli from leaf and hypocotyl explants on different media combinations for regeneration

| Sl.No. | Media composition | Genotype | Response |
|---|---|---|---|
| 1. | MS + BAP 1 mg $l^{-1}$ + 2 % sucrose | C 152 | NR |
| | | CO 4 | NR |
| | | CO 6 | NR |
| 2. | MS + BAP 1.5 mg $l^{-1}$ + NAA 0.5 mg $l^{-1}$ + 2% sucrose | C 152 | NR |
| | | CO 4 | NR |
| | | CO 6 | NR |
| 3. | MS + BAP 2.0 mg $l^{-1}$ + NAA 1.0 mg $l^{-1}$ + 2% sucrose | C 152 | NR |
| | | CO 4 | NR |
| | | CO 6 | NR |
| 4. | MS + BAP 2.5 mg $l^{-1}$ + NAA 1.5 mg $l^{-1}$ + 2% sucrose | C 152 | NR |
| | | CO 4 | NR |
| | | CO 6 | NR |
| 5. | MS + BAP 3.0 mg $l^{-1}$ + NAA 2 mg $l^{-1}$ + 2% sucrose | C 152 | NR |
| | | CO 4 | NR |
| | | CO 6 | NR |

NR = No Response

# *DISCUSSION*

# CHAPTER 5

## DISCUSSION

Cowpea (*Vigna unguiculata* (L.) Walp., is an important legume commonly cultivated in South India. It provides more than half the amount of plant proteins in human diets (Rachie, 1985). Among the numerous pathogens several viruses are known to infect cowpea. Viral diseases devastate this crop and are a major constraint to increased productivity wherever they are cultivated. Detailed studies for the identification of the different viruses causing these diseases have not been done in many cases. Characteristics of these viruses reported from different places in India differ from each other in many aspects. Thus, an adequate knowledge of the virus occurring in the main cowpea growing areas is a prerequisite for the effective control of the disease in question.

More than 20 viruses have been reported on cowpea from different areas of the world (Thottappilly and Rossel, 1985; Mali and Thottappilly, 1986; Brunt *et al*., 1990). Only very little attempt has been made so far to identify the cowpea mosaic virus occurring in South India. In the present investigations, an attempt has been made to identify the virus and to formulate a suitable control measure for the disease. The results of the study are discussed hereunder.

## 5.1. SYMPTOMATOLOGY

The symptoms caused by the virus under study were similar to those produced by aphid-borne mosaic disease. Main symptoms of the disease were dark green vein-banding, leaf distortion, blistering, severe mosaic and stunting similar to those described by Lovisolo and Conti (1966). Mali and Kulthe (1980) reported that the symptoms on primary leaves were mild mosaic, followed by irregular mosaic or yellow mottle, puckering, slight distortion and arching of trifoliate leaves. They also observed slight stunting of the plants. In the present studies, it was observed that the initial symptoms appeared on the leaf produced 14 days after inoculation in the form of mild vein clearing. Typical mosaic mottling with dark green and light green patches appeared on the leaves produced later on. A few leaves showed prominent vein banding and occasionally dark green blisters. A comparison of the symptoms of the cowpea aphid-borne mosaic disease reported by the earlier workers with those of the mosaic disease found in Tamil Nadu showed that the symptoms described from different places were more or less similar.

## 5.2. THE VIRUS

CAMV particles were characterised by flexuous, filamentous rods with a calculated mean length of 750 nm (Bock and

Conti, 1974; Kaiser and Mossahebi, 1975; Lima *et al.*, 1979). The size of the particles were 765 nm when partially purified preparations from systemically infected cowpea plants were analysed under TEM (Fischer and Lockhart, 1976). Further studies on the virus causing mosaic disease of cowpea by aphid species also confirmed the presence of similar flexuous rod shaped particles in cowpea leaves (Mali and Kulthe, 1980b; Patel and Kuwate, 1982; Chang and Kuo, 1983; Thottappilly and Rossel, 1992).

In the present study using electron microscope, the particles were found to be flexuous, filamentous measuring a length of approximately 750 nm, which confirms with findings of the earlier workers mentioned above. However, some particles were smaller in size which might be due to the fragmentation of virus particles during specimen preparation and storage. Degradation of chicory yellow mottle virus upon storage of infected tissue or purified virus suspension at -25°C was reported by Quacquarelli *et al.* (1972). Therefore, this aphid transmitted filamentous virus could be classified under potato virus Y group based on the classification of Brandes and Bereks (1965).

## 5.3. TRANSMISSION OF THE DISEASE

The CAMV was found to be readily transmitted by mechanical means and produced the characteristic symptoms on

cowpea cultivars systemically within 2-4 weeks after inoculations. Several investigators (Patel and Kuwaite, 1982; Atiri and Thottappilly, 1984; Atiri *et al.*, 1984; Revathi, 1991; Patil and Gupta, 1992b; Thottappilly and Rossel, 1992; Nain *et al.*, 1994) have reported that the virus was also transmitted by sap inoculation.

The virus could be transmitted more efficiently using different buffers. The results in Table 1 indicate that maximum infection was obtained when 0.1M potassium phosphate buffer (pH 7.5) was used as the extraction medium, followed by 0.1M sodium phosphate buffers, (pH 7.4 and 7.0) 0.1M cacodylate buffer, pH 7.0 and 0.1M citrate phosphate buffer (pH 7.0). The virus appeared to be more stable in potassium and sodium phosphate buffer (Table 2), which sustained the infectivity even up to 24 h at room temperature. Borate and tris-HCl were found to be very weak sources of buffers helpful in the transmission of CAMV.

The results on seed transmission indicated that the virus was also transmitted through seeds at different levels in different cultivars. CAMV was the only virus, of four affecting cowpeas naturally occurring in Iran was seed-borne, although all were aphid-transmitted (Kaiser *et al.*, 1968; Kaiser, 1972). They also reported that the seed transmission of CAMV in susceptible cowpea lines ranged upto 39.8 per cent. In the present study the

level of seed transmission ranged from 5.77 to 15.38 (Table 3). This finding was in accordance with the earlier reports (Jeyanandarajah, 1992; Patil and Gupta, 1992a; Thottappilly and Rossel, 1992; Bashir and Hampton, 1993, 1994).

Studies on the insect transmission of CAMV was conducted using A.craccivora, A.gossypii, M.persicae, B.tabaci and C.trifurcata. Among these insects, A.craccivora was found to transmit CAMV in a very efficient manner giving a maximum of 93.33 per cent transmission while M.persicae and A.gossypii gave 30 and 26.67 per cent respectively. The whitefly B.tabaci and the beetle C.trifurcata failed to transmit the virus (Table 4). Nain et al. (1994) also reported that A.craccivora transmitted CAMV (88 per cent) more efficiently when compared to other vectors tested.

Tsuchizaki et al. (1970) proposed the name cowpea aphid-borne mosaic virus (CAMV) be used for cowpea viruses of the potato virus Y group in order to avoid confusion in nomenclature between the aphid-borne filamentous and the beetle-transmitted spherical viruses that naturally infect cowpeas.

CAMV was readily transmitted in a non-persistent manner by A.craccivora, Agossypii, Aspiraecola, A.citricola, A.medicaginis, M.euphorbiae, M.persicae, R.maidis and C.palmae

(Atiri et al., 1984, 1986; Thottappilly and Rossel, 1992; Nain et al., 1994). The whitely B.tabaci and the beetle C.trifurcata were not reported to be the vectors of CAMV, but they were included in transmssion trials in order to confirm whether the disease was of CMMV and CYMV. The consensus of information from the present and former studies support the generalization of Aphid spp.(A.craccivora, A.gossypii and M.persicae) as efficient vectors of CAMV.

## 5.4. VIRUS-VECTOR RELATIONSHIP

The virus-vector relationship was studied using the most efficient vector, A.craccivora. Acquisition access period, inoculation access period, influence of starvation before acquisition and inoculation feeding, minimum number of aphids required for effective transmission and retention of infectivity by the vector were investigated.

In the course of experiments that were designed to verify the effect of different acquisition periods, it became apparent that with a minimum acquisition period of 5 minutes the virus could be transmitted to healthy plants. As the acquisition feeding period was increased the per cent transmission also increased and the maximum transmission of 90 per cent was obtained when an acquisition periods of 30 minutes and 1 h were

given. After this there was a steady decline. Several workers have also demonstrated that CAMV was transmitted during probes of only a few seconds (Kaiser and Mossahebi, 1975; Mali and Kulthe, 1980a; Atiri, 1982; Guo et al., 1984; Santos et al., 1984; Atiri and Thottappilly, 1984; Murphy et al., 1987; Li et al., 1990; Ndiaye et al., 1993; Roberts et al., 1993).

For most non-persistent viruses following aphid acquisition probing on infected plant at different inoculation periods will give an idea about the efficacy of inoculation access period on the transmission of the virus by aphids. The results of the present experiment using CAMV were found similar to those obtained by the earlier workers (Govindaswamy et al., 1970; Murugesan and Janaki, 1972). The minimum inculation feeding period of CAMV in the present study was found to be one minute (Table 6). The per cent transmission was found to increase with the increase in inoculation access period and reached the maximum of 80 per cent when 30 minutes inoculation feeding was given. Thereafter the per cent transmission declined and it reached 40 per cent with 24 h inoculation feeding. This agrees with the observations of Nariani and Kandaswamy (1961) and Murugesan and Janaki (1972). On the other hand, the present results differ from those of Atiri et al. (1984) who stated that CAMV was transmitted during probes of a few seconds.

Investigations on the influence of starvation before acquisition and inoculation feedings showed that preacquisition starvation caused a steady increase in the per cent transmission up to one hour fasting and there was considerable reduction in transmission thereafter. Preinoculation starvation caused a reduction in the transmission efficiency. Maximum infection of 90 per cent was obtained without starvation. Similar results were obtained by Nariani and Kandaswamy (1961) and Murugesan and Janaki (1972). Revathi (1991) observed that maximum transmission was obtained with CAMV when aphids were given a fasting period of 15 and 10 minutes respectively before acquisition. Nain et al. (1994) could successfully transmit CAMV when nymphs of aphids were given one hour fasting prior to acquisition access period. The results of the present investigtion also agree with this observation (Tables 7, 8). The difference in the efficiency of transmission when different periods of starvation before acquisition and inoculation access period to transmit CAMV may be due to the nature of aphid probing and environmental factors.

Experiments on the minimum number of aphids required for successful transmission of CAMV were also worked out. It was found that a single viruliferous aphid could successfully transmit the virus. Similar observation was made in the case of transmission of CAMV by Sharma and Varma (1977). Maximum infection of 70 per cent was obtained in the present study when 5 and

10 aphids were used for the transmission. Several workers have also demonstrated a correlation between the abundance of aphid vectors and virus spread (Thresh, 1974).

Investigations on retention of infectivity by A.craccivora revealed that the vector lost its infectivity after 2 h in all the 6 series of experiments carried out except when 1 h 30 minutes feeding interval was given wherein the infectivity was retained even after two hours. This indicates that in most cases A.craccivora retained CAMV for only about 2 h which implies that the viruliferous nature of the virus was lost after 2 h of successive feeding.

5.5. HOST RANGE OF THE VIRUS

Nariani and Kandaswamy (1961) found that the host range of the type strain of CAMV in greenhouse inoculation studies was confined to the family Leguminosae. Bock (1973) described a wide host range spread over to 19 leguminous and 11 non-leguminous hosts. Several other workers found that the host range included several families besides Leguminosae (Govindaswamy et al., 1970; Bock and Conti, 1974; Khatri and Singh, 1974; Ramiah, 1978; Mali and Kulthe, 1980; Patel and Kuwaite, 1982; Boswell and Gibbs, 1983; Nain et al., 1994; Thenmozhi, 1994). Similar results were obtained in the present investigations also.

Out of the 48 plant species tested, belonging to 11 families, 13 plant species showed positive reaction with CAMV. CAMV however, was not found to infect other food legumes tested.

Boswell and Gibbs (1983) reported that 10 plant species viz., G.globosa, C.amaranticolor, C.quinoa, C.sativus, O.basilicum, G.max, P.vulgaris, P.sativum, T.foenum-graecum and P.hybrida could be used as diagnostic hosts of CAMV. They also found that V.unguiculata was the only angiosperm species infected naturally and no non-angiosperm species were infected naturally. They stated that the experimental host range was intermediate (3-10 families susceptible). In the present investigations the diagnostic species C.amaranticolor, G.globosa, C.quinoa, O.basilicum, G.max, P.vulgaris and P.sativum were tested. The symptoms produced in the present study also showed marked similarity with their findings. Therefore it is concluded that the present virus isolate may be a different strain of CAMV.

## 5.6. ANATOMICAL STUDIES

Anatomical studies were undertaken in order to study the nature of damage caused by the virus. The histopathological studies showed marked disorganisation of mesophyll, palisade, parenchyma and less frequently, epidermal cells as evident from the CS of leaves from diseased plants (Plate 26f). The LS of

apical meristem of diseased samples also showed retarded growth of meristem and leaf primordia (Plates 26b to 26d). This indicates that there was significant effect on the growth of the plants infected with the virus. On the contrary, Jeyanandarajah and Burnt (1993) reported that the virus, did not induce any conspicuous cyto-pathological effects in infected cells with CMMV except for brush-like inclusions consisting apparently of virus particles.

## 5.7. DETECTION OF CAMV

The detection for the presence and assay of pathogen population are basic requirements for the elimination of the pathogen at the earliest to minimise the disease incidence and its subsequent spread. Many workers have developed simple and sensitive serological methods for the detection of viruses in infected plants and vectors involved in their spread. In the present investigation an attempt has been made to develop reliable methods of diagnosis of the virus in plants and the aphid vectors.

### 5.7.1. Detection of CAMV by DAC-ELISA

Serodiagnosis of cowpea viruses and identification using different immuno techniques have been well documented.

DAC-ELISA was performed to detect and identify the virus causing mosaic disease in cowpea. Antisera raised against CAMV (polyclonal and monoclonal) and four BICMV monoclonals were used in the present study. BICMV and CAMV were found to be serologically distantly related but were distinct potyviruses (Taiwo _et al_., 1982). Lima _et al_. (1979) and Dijkstra _et al_. (1987) reported that they were serologically closely related. Purciful and Gonsalves (1985) pointed out that they were distant serologically. Huguenot _et al_. (1993) clearly demonstrated that CAMV and BICMV were two distinct members of the potyvirus family based on the monoclonal antibodies approach. They specifically detected using a mixture of monoclonal antibodies.

In the present investigation, the highest reactivity was found with CAMV polyclonals and monoclonal antibodies. However the BICMV monoclonals showed only very low reactivity (Table 12). The results reveal that both the viruses (CAMV and BICMV) are present in cowpea grown in Coimbatore.

### 5.7.2. Detection of CAMV by NCM-ELISA

In the present investigation an attempt was made to detect the presence of the virus in plants and aphid vectors based on the procedure developed by Lizarraga and Fernandez-Northecote (1989). The results indicated that the virus could be detected at very high dilution (1:1000) in sap from infected

cowpea plants and the reaction was positive even in body fluids from single viruliferous aphid. This highly sensitive technique could be effectively used under field conditions. Enzyme-linked immunosorbent assay on nitrocellulose membranes (Bode et al., 1984) was used for the evaluation of virus resistant germplasm exposed in the field.

### 5.7.3. Transmission electron microscopy (TEM) and Immunosorbent electron microscopy (ISEM) of CAMV

The results clearly indicated that the CVC from infected leaves showed the presence of fluxuous, filamentous particles which were about 750 nm. ISEM also proved that both the antisera (CAMV and Potyvirus group) could trap the virus. Trapping caused immuno purification of the virus on the grid. The net result was the binding of virus particles with a cleaner background. Since the present study was aimed to know the identity of the virus rather than its detection, the ISEM clearly indicated that the virus isolate belongs to CAMV potyvirus group.

## 5.8. PHYSIOLOGY AND BIOCHEMISTRY OF VIRUS INFECTED PLANTS

### 5.8.1. Effect of total chlorophyll content

The effect of CAMV on the total chlorophyll content of cowpea plants indicated that there was significant reduction in inoculated susceptible varieties, C 152 and CO 4. The maximum

reduction was observed at 40 days after inoculation. There was no alteration in chlorophyll content in resistant varieties due to infection (Table 13). Similar cases of reduction in chlorophyll content in plants infected with cowpea mosaic virus has been reported (Ramiah, 1978; Tripathi et al., 1987; Mayoral et al., 1989). Rao et al. (1989) found that the total chlorophyll, chlorophyll a and b increased upto 60 days and then declined. They correlated the yield loss with the reduction in rate of photosynthesis. The present study also confirms the findings of the earlier workers.

5.8.2. Effect on total carbohydrate content

The present study revealed that there was a reduction in the level of carbohydrate content in susceptible varieties inoculated with CAMV when compared to healthy control. There was also gradual increase in the total carbohydrate content in both the susceptible varieties with increase in age of the plant (Table 14). Such lower levels of carbohydrate have been reported in cowpea by many workers (Khatri and Chenulu, 1969; Ramiah, 1978; Singh and Singh, 1984; Mayoral et al.,1989). Narayanasamy and Ramakrishnan (1966) suggested that the reduction in the level of carbohydrates might be due to the breakdown of the carbohydrates which have to provide the substrates for the accelerated respiration in virus infected plants. This observation justify

the observed lower level of carbohydrate in susceptible varieties. Ramiah (1978) also found the similar pattern in susceptible cultivars tested.

5.8.3. Effect on total soluble protein

The results in Table 15 indicates that there was an increase in the level of total soluble protein when inoculated with CAMV, than in uninoculated control. Inoculation of resistant variety with CAMV did not cause much difference in total protein content. Ramiah (1978) found that the total nitrogen content was higher in the inoculated cowpea plants than that of comparable healthy plants. Similar supporting evidences of increase in total protein content in virus infected plants have been reported by many authors (Singh *et al*., 1978; Mali *et al*., 1980; Singh and Singh 1981; Narayanasamy and Ganapathy, 1986). Dumas *et al*. (1988) found increased synthesis of soluble proteins in Petunia leaves hypersensitive to TMV. The reason they found for the higher protein content in infected susceptible plants was due to the synthesis of virus coat protein and other virus associated non-structural proteins.

The synthesis of three new virus related proteins during infection of cowpea with CAMV and application due to AVPs has been demonstrated in Plate 34. This is in agreement with the studies of protein synthesis in other virus infected plants, which revealed the formation of many virus-related polypeptides. This is also true in the case of AVP treatment supported by earlier workers. Cowpea mosaic virus infection gives rise to the formation of virus-specific cytopathic structures which are presumed to have an essential function in virus RNA replication (De Zoeten *et al*., 1974; Hibi *et al*., 1975). The synthesis of these structures, however, might have altered the pattern of protein synthesis and stimulated the synthesis of host proteins. This may be the possible reason for the enhanced synthesis of proteins in plants inoculated with CAMV and treated with AVPs.

### 5.8.6. Electrophoretic analysis of isozymes

Electrophoresis of protein extracts from plant tissue, using different kinds of support media and buffer systems, allows separation of the multiple forms of enzymes (isozymes) on the basis of charge and/or molecular size. The resulting polymorphisms are useful as genetic markers. Isozyme analysis is a powerful technique for estimating genetic variability, identifying cultivars and germplasm accessions (Asiedu, 1992).

The results presented in Table 16 and Fig. 5 showed that there was distinct polymorphism in isoPOs between resistant and susceptible lines screened from the germplasm. This polymor phism can be used as a marker for identification of resistant lines. Three isoforms of peroxidase at Rf values 0.33, 0.45 and 0.71 were detected in all the six resistant genotype with particular enhancement to the isozyme band at Rf 0.33. One apparently novel isozyme was detected at Rf 0.45 for the six resistant genotypes.

Isozyme analysis has had many useful applications in plant genetics and breeding (Tanksley and Orton, 1983; Asiedu et al., 1990; Kephart, 1990). Within the limits of the correlation for variability at isozyme loci and variability at loci for agronomic traits, isozyme polymorphisms have been useful indicators of the diversity of genotypes (Brown et al., 1978; Asiedu et al., 1990). Identification of cultivars and germplasm accessions through isozyme analysis offers far more precision than using morphological descriptors alone.

5.8.7. Estimation of nucleic acids

The present investigation on the changes in nucleic acid contents showed that there was a gradual increase in RNA content in susceptible cultivars following inoculation with CAMV.

The level of RNA in resistant cultivar, CO 6 was found to be higher in both inoculated and healthy plants. This was in agreement with the findings of Lee *et al*. (1975) where they found that the level of RNA was 5.6 times more in plants infected with cowpea mosaic virus when compared to healthy control. Ramiah (1978) also observed that the RNA content of cowpea plants maintained a higher level at 20 and 40 days after inoculation with CAMV. Sastry and Nayudu (1988) while working with tobacco ring spot virus in cowpea arrived at similar conclusion and reported higher content of RNA in infected plants. Similar trend was observed in *Dolichos lablab* infected with yellow vein mosaic virus (Hossain and Haider, 1992).

The results of the present study showed that the level of DNA content was low in inoculated susceptible cultivars when compared to uninoculated control. Ramiah (1978) while investigating the level of DNA in cowpea plants infected with CAMV revealed that there was a significant increase in the DNA content in susceptible cultivar when compared to resistant variety. Sastry and Nayudu (1988) also observed the same trend which was not in agreement with the findings of Hossain and Haider (1992) where they observed a decrease in total DNA content in infected plants. The evidence presented in the present investigation agrees with the latter findings.

5.8.8. Assay of enzyme activities

Investigations on the changes in the activities of peroxidase, polyphenol oxidase, phenylalanine ammonia-lyase and chitinase clearly indicated that there was significant increase in the activities of these enzymes in inoculated plants. Treatment of cowpea plants with BSLE also found to accelerate the enzyme activities. Treatment of BSLE followed by inoculation with CAMV also enhanced the enzyme activities.

Khatri and Chenulu (1970) found higher peroxidase activity in inoculated susceptible varieties. They also found an increased catalase activity in cowpea plants infected with cowpea mosaic virus. Similar trend was observed in cucumber plants infected with cucumber mosaic virus 2 and 2A (Serova *et al*., 1972). Lee *et al*. (1975) found that the enzyme aspartate transcarbamylase activity in diseased plants increased nearly by four times than the healthy plants. Several workers have studied the activity of three common enzymes *viz*., peroxidase, polyphenoloxidase and ribonuclease and have outlined quantitative changes in activity before, during and after virus induced necrosis (Loebenstein, 1972; van Loon, 1982; Wagih and Coutts, 1982a). Systemic resistance in hypersensitive tobacco to virus infection was also considered to be due to higher activities of catalase, peroxidase and polyphenol oxidase enzymes (van Kammen and

Brouwer, 1964; Simons and Ross, 1971). Verma and Prasad (1987) found that the aqueous leaf extracts of Clerodendrum aculeatum induced systemic resistance to sunnhemp rosette virus in Cyamopsis tetragonoloba which was correlated with the altered physiology of the test plant. They established that the induced resistance was attributed to the enhanced activity of catalase, peroxidase and polyphenol oxidase enzymes following treatment with the leaf extract. These oxidative enzymes at their peak activity oxidises phenolics to quinones, which in turn inactivate the virus (Hampton and Fulton, 1961; Mink and Saksena, 1971). The ele-vated activity of polyphenol oxidase and peroxidase would lead to an accumulation of phenol-oxidized products and quinones, which inactivated the virus (Verma and Prasad, 1987).

5.8.9. Changes in phenol content

The present investigation revealed that the resistant line showed a significantly higher total phenol content when compared to the susceptible lines at all stages of analysis. Following inoculation there was an enhancement of total phenol content in both susceptible and resistant lines. It showed a peak at third day after inoculation. The OD phenol content also showed similar trend in the present study. Padma et al. (1976) found higher phenol content in infected seeds of cowpea. Ramiah (1978) observed that the total phenol content increased in the

CAMV inoculated leaves of the susceptible cultivars of cowpea. Enhanced level of phenol content has been observed in hypersensitive cowpea primary leaves infected with tobacco ring spot nepovirus (Sastry and Nayudu, 1988). Kato et al. (1993) extracted and characterised two phenolic compounds from cowpea leaves infected with cucumber mosaic virus. They showed their enhanced level during infection. Khem (1995) found that the total phenolics of susceptible bhendi plants increased substantially upto 10 days after inoculation with OYVMV (Okra yellow vein mosaic virus). It showed a decreasing trend thereafter. The OD phenol also showed similar trend.

5.9. MANAGEMENT OF CAMV

Virus diseases are a major limiting factor in the production of cowpea. One of the viruses, cowpea aphid-borne mosaic virus (CAMV) is a seed-borne disease which is systemic in susceptible cowpea cultivars. So far no effective chemicals are available for the control of systemic diseases caused by viruses. Modern control of virus and virus-like diseases are achieved by integrated systems involving different principles of plant disease control including the use of virus-free planting stock and seedling program, use of antiphytoviral chemicals, antiviral principles from plant origin, control of vectors using insecticides and use of resistant cultivars.

In the present investigation an attempt was made to manage the disease using antiphytoviral chemicals and antiviral principles from plants under glasshouse conditions and a field trial was undertaken using four promising plant extracts, two oils (plant origin) and an insecticide (monocrotophos) to find their efficacy in the management of CAMV disease.

5.9.1. Management using virus inhibitory chemicals

The results indicated that pre-inoculation sprays of barium chloride, ammonium molybdate and copper sulphate (1000 ppm) and thiouracil (500 ppm) gave an inhibition of more than 80 per cent.

Use of antiphytoviral chemical for the control of virus diseases have been developed during the recent years by many investigators. Pennazio _et al_. (1983) found that sodium salicylate reduced both the size and viral antigen content of non-self limiting necrotic lesions produced by tobacco necrosis virus (TNV). They found that the antiviral effect of salicylate occurred at concentrations close to the limits of toxicity. It has previously been demonstrated that salicylates decrease lesion number and size in different host-virus systems (White, 1979; Antoniw and White, 1980; Pennazio and Redolfi, 1980; van Loon and Antoniw, 1982).

In the present investigation also salicylic acid (1000 ppm) gave an inhibition of 72.82 per cent and acetyl salicylic acid (ASA; 1000 ppm) gave an inhibition of 59.41 per cent. The mode of action of ASA and salicylates in general was unknown ealier. Studies support that the reduction of virus multiplication may be consequent to the alterations of the host cell plasma membrane induced by ASA (Conti et al., 1988). Such alterations lead to a loss of cell turgidity, and less turgid cells are less susceptible to viral infection (Mills and Wood, 1984). The plasma membrane alterations lead to changes in cell permeability (Wagih and Coutts, 1982b; Pennazio et al., 1987) and may be at the basis of the accumulation of PR-proteins found in the intercellular spaces after administration of salicylate (Ohashi and Matzuoka, 1987). This might be another cause of the reduced virus spread as it seems that PR-proteins, whose synthesis is induced by ASA and salicylates in general, are involved in plant resistance to virus infection (Antoniw and White, 1980; Pennazio and Redolfi, 1980; Matzuoka and Ohashi, 1986).

Results of the application of antiphytoviral agents such as ribavirin, 2,4-dioxohexahydro-1,3,5-triazine (DHT) compounds having the azine structure and a membrane lipid analogous compound against tobacco virus have been reported (Schuster, 1986). White et al. (1986) found that the antiviral chemicals viz., 2-thiouracil, DHT, barium chloride and manganese chloride

induced PR-proteins which in turn enhanced the resistance to TMV infection in tobacco. DHT and 2-thiouracil interfere with nucleic acid metabolism. The inhibitory effects of 9-(2,3-dihydroxypropyl) adenine and 3-(adenin-9-yl)-2-hydroxy propanoic acid 2-methyl propylester on potato virus X replication was reported by Schuster and Holy (1988). They found that the latter compounds inhibited virus replication more effectively at lower concentration range. The mode of action of the chemical they stated was inhibition of a late event in the replicative cycle of PVX (viral mRNA synthesis and/or maturation or early synthesis of viral protein which corresponds to viral mRNA methlation).

Bauer et al. (1993) found that 1-($\alpha$ -carboxyalkyl)-4,5-dimethylimidazol-3-oxides showed antiphytoviral activity against red clover mottle virus and alfalfa mosaic virus in systemically infected host plants. They also reported that membrane modifications induced by these compounds may be the reason for their antiphytoviralactivity because some replication process occurred on membranes. Kovalenko et al. (1993) observed that mannan sulphate (MS) induced resistance to TMV in hypersensitive plants. They also found that MS-induced resistance in tobacco was accompanied by a marked increase of lytic processes in cells and decreased the total (acidic and alkaline) protein content. In addition new protein compounds including PR-proteins

and antiviral substances of the inhibitor of virus replication (IVR) type, appeared in treated plants.

5.9.2. Management using plant products

Among the 25 plant species tested, leaf extracts from C.annum gave complete inhibition. Leaf extracts from B.rubra, C.bonplandianum, I.carnea and M.jalapa were found to be strong inhibitors of CAMV. Leaf extracts when mixed with the virus inoculum before inoculation also gave significant inhibition of CAMV.

The antiviral principle (AVP) have been found to act differently in different host-virus system. Ragetli and Weintraub (1962a) suggested that AVP might act as a competitive inhibitor of virus infection by occupying the infective sites which become unavailable for virus particles for infection and multiplication. Owens et al. (1973) found that AVP might block the messenger activity of the virus RNA, affecting virus replication. Verma and Awasthi (1979) while investigating the antiviral activity of B.diffusa root extracts reported that the inhibitory effect may be attributed to blocking of host cell receptors or to interference of virus synthesis in the host cells.

Ribosome-inactivating proteins (RIPs) are another class of plant protein, which have been shown to inhibit the infectious plant viruses (Stevens *et al*., 1981). The best characterized RIP is the pokeweed antiviral protein (PAP) which has been purified from the leaves and seeds of *Phytolacca americana* (Irvin *et al*., 1980; Chen *et al*., 1991). The presence of RIPs in *M.jalapa* have also been identified (Habuka *et al*., 1991). Owens *et al*. (1973) suggested that PAPs antiviral activity may be due to the inhibition of the viral protein synthesis and demonstrated that PAP inhibited the *in vitro* synthesis of polypeptides by ribosomes from cowpea and wheat germ. Lodge *et al*. (1993) observed that PAP entered the host cell along with the virus and prevented the translation of virus RNA. They also suggested that PAP might have bound to the virus or to the cell wall component which prevented the entrance of the virus into the cell.

Development of resistance to viruses in AVP treated plants appeared to be linked to the formation of new proteins (Rao *et al*., 1985; Awasthi *et al*., 1987). Kubo *et al*. (1990) found that the proteinaceous inhibitor from *M.jalapa* was able to penetrate into leaf cells and get translocated to long distance in sufficient amount to block the virus infection directly. They observed that the systemic effect of *Mirabilis* antiviral protein (MAP) might be a host-mediated phenomenon.

5.9.3. Nature of AVPs

The present investigation revealed that the protein fraction of leaf extracts from M.jalapa, B.spectabilis, P.chilensis and I.carnea effectively inhibited the CAMV infection. Many other workers also have reported the proteinaceous nature of the AVPs involved in the inhibition of virus diseases. Protein or glycoproteins having inhibitory activity against plant viral infection were obtained from several higher plants, such as P.americana (Irvin, 1975), Dianthus caryophyllus (Ragetli and Weintraub, 1962a,b), Chenopodiales (Smookler, 1971), B.diffusa (Verma and Awasthi, 1979), Yucca recurvifolia (Okuyama et al., 1978), S.vulgare and C.nucifera (Narayanasamy and Ramiah, 1983), B.spectabilis (Verma and Dwivedi, 1984), Pseudoranthemum atropurpureum (Verma and Khan, 1985). MAP was in common with protein as that in P.americana and Y.recurvifolia in respect of being lysine rich, basic protein (Takanami et al., 1990). Krishnamoorthi (1994) also reported that the protein fractions of the extracts from M.jalapa, V.nigundo and L.leucocephala effectively reduced the urdbean leaf crinkle virus (ULCV) infection when compared to the non-protein fraction.

In the present investigation, partially purified Bougainvillea antiviral protein (BAP) when sprayed on cowpea seedlings altered the physiology of both resistant and

susceptible varieties. The enzymes PO, PPO and PAL displayed a higher level of activity during early stages of application. Similar enhanced activities of enzymes have been reported by Verma and Prasad (1987) when leaf extracts of Clerodendrum aculeatum was sprayed on C.tetragonoloba. They found that the induced resistance was attributed to the enhanced activity of catalase, PO and PPO following treatment with the leaf extract.

5.9.4. Management of CAMV disease under field condition

In the present investigation using plant extrats, oils and insecticide for managing the CAMV revealed that the treatments showed marked reduction in the per cent infection besides delaying symptom expression (Tables 33 to 37). The treatments also improved the height of the plants, number of leaves, length of roots, fresh and dry weights of the plants at different stages of analysis, which were clearly demarcated over diseased control. The population of aphids present in treated plants was much lower than the control. A significant variation in grain yield was observed among treated plants. The maximum yield was obtained BSLE treated plots followed by monocrotophos and MJLE treatments. The lowest was recorded in control.

Verma and Kumar (1980) noticed a marked inhibition of CMV by M.jalapa leaf extract in treated cucumber and melon

plants. It also improved the height and flowering. The population of aphids was considerably reduced when compared to control. Murthy (1982) found that M.jalapa strongly reduced the TMV multiplication in the treated N.glutinosa plants. It was also found that per cent infection was reduced and the incubation period was prolonged in plants treated with P.chilensis, D.metel, C.nucifera and S.vulgare. Narayanasamy and Ramiah (1983) reported that leaf extracts of S.vulgare reduced the number of lesions produced by TSWV on cowpea leaves when mixed with inoculum. Pillayarsamy et al. (1988) reported that leaf extracts of M.jalapa, B.spectabilis, L.leucocephala, Tribulus terrestris, Achyranthes aspera, Alternanthus echinata, Phyllanthus nirurii, Pisona alba, Prosopis sp., D.metel and Acalypha indica reduced the CAMV infection. Inhibition of potyvirus using leaf extracts from B.spectabilis, M.jalapa, P.americana, Crinum augustum, C.annum and D.stramonium have been reported by many authors (Durate et al., 1990; Fahmy and Mohamed, 1990; Molina and Leon, 1991). Revathi (1991) also found that Aegle marmelos and V.nigundo gave the maximum inhibition of CAMV disease under field condition. antiphytoviral activity of B.diffusa, B.spectabilis, C.aculeatum and S.vulgare against CAMV was reported by Sadasivam et al. (1991). Raghupathy (1995) found that leaf extracts of V.nigundo, Eucalyptus globulus, Cynodon dactylon, Pongamia glabra, Acacia arabica, D.metel, Thuja occidentalis, Ocimum sanctum, Coleus parviflorus, Mentha piperita and Dioscorea alata were effective

in reducing TLCV (tomato leaf curl virus) infection. Khem (1995) reported the antiviral property of leaf extracts of P.chilensis and B.spectabilis under field condition.

In the present investigation also leaf extracts from B.spectabilis, M.jalapa, I.carnea and P.chilensis showed inhibition of CAMV disease. BSLE and MJLE were found to be superior which gave an average inhibition of 88.14 per cent followed by ICLE and PCLE with an average inhibition of 82.14 per cent at 90 days after sowing. MJLE was superior in the management of aphid population which gave an inhibition of 71.42 per cent followed by BSLE, PCLE and ICLE. Narayanasamy (1984) and Verma and Khan (1985) viewed that the AVP might induce the resistance by activating the host defence system.

In the present experiment two non-edible oil and a systemic insecticide were used for the management of aphid population to prevent the secondary spread of the virus. Maximum inhibition (cent per cent) was found when the systemic insecticide was sprayed followed by neem oil (93.66 per cent) and mahua oil (91.75 per cent) at 60 DAS. Use of systemic insecticide was found to be more efficient in the control of CAMV disease under field condition.

The efficacy of different oil formulations and insecticides for the management of vectors were reported by many authors. Hein (1972a, b) reported the inhibition of mineral oil and plant oils of mechanically transmitted viruses and prevented the aphid transmission. Dubey and Nene (1975)found that the viruliferous aphids lost the virus while probing on cowpea plants sprayed with emulsion of castor, light paraffin and emulsifiable oils. They also observed that the spread of CAMV by aphids from sprayed infected to sprayed healthy was appreciably delayed due to oil sprays. Raghupathy (1989) found that one per cent neem oil spray contained the spread of soybean yellow mosaic virus and was on par with monocrotophos. Revathi (1991) also reported that neem oil one per cent significantly reduced CAMV incidence under field condition and gave maximum yield. Gibson and Rice (1986) could find that pyrethroids effectively controlled PVY and decreased the acquisition of non-persistently transmitted viruses. Atiri et al. (1987) found that both cypermethrin (at 100-200 mg a.i.$l^{-1}$) and deltamethrin (at 75-150 mg a.i. $l^{-1}$) restricted the acquisition and inoculation of CAMV. This may be because virus transmission was affected by changes in the behaviour of the aphids. They also found that rapid knockdown effect of pyrethroids incapacitated aphids before they could transmit the virus.

Gibson et al. (1982) found that organophosphates and carbamatè aphicides restrict secondary spread of semipersistent and persistent virus but seldom kill aphids fast enough to control secondary spread or primary introduction of non-persistent viruses. Monocrotophos used in the present investigation eventhough gave promising results in the management of CAMV its use under field conditions may be restricted for the control of aphids. Moreover Rice et al. (1983) demonstrated that organophosphate has the potential of increasing the spread of potyviruses. Therefore, fast acting synthetic pyrethroids which have rapid knockdown properties (Briggs et al., 1974), may control the CAMV virus (Atiri et al., 1986).

Glasshouse trials using foliar sprays of plant extracts and insecticide also showed significant reduction in the number of infected plants besides slightly increasing the incubation period of the virus in plants.

From the results available in this study and also from those obtained by other workers, it is evident that the effect may be attributed to the inhibition of infection by some constituents present in leaf extracts used in the experiments. The possibility of systemic translocation of active antiviral factors in plant extracts also cannot be ruled out. The oils and insecticide might have acted on the aphid population by repellent and

aphicidal action. Consequently the number of virus particles which entered the plants might have been reduced. Earlier reports have also established that foliar application of plant extracts induce defence mechanism in plants, which inhibits the virus multiplication. From this it may be inferred that they might have acted predominantly on the inhibition of virus infection. To pinpoint the precise mechanism of action of the plant products some more indepth study is necessary.

## 5.10. SCREENING FOR SOURCES OF RESISTANCE TO CAMV IN COWPEA GERMPLASM

The best single control method is the use of resistant varieties. The first step towards developing such varieties is large-scale screening and selection among germplasm collections of the crop, followed by breeding new crop genotypes, where possible, that combine virus resistance with other useful traits (Thottappilly and Rossel, 1992). Singh and Ntare (1985), Thottappilly and Rossel (1985) and Singh *et al*. (1992) reviewed the development of cowpea lines with multiple resistance to different viruses to different viruses infecting cowpeas. Several improved cowpea lines with resistance to as many as five viruses have been developed. Eight varieties *viz*., IT 81D-1137, IT 82E-16, IT 82D-889, IT 83S-818, IT 83D-442, IT 84D-449, IT

85F-867-5 and IT 85F-2687 were developed as resistant lines of cowpea to CAMV from IITA, Ibadan, Nigeria.

In the present investigations also numerous sources of resistance to CAMV were found in the 332 cowpea lines that were included in the screening trials. Some of these lines (40) showed high levels of resistance to CAMV in glasshouse inoculation trials. Seventy eight showed tolerant reaction, 90 were susceptible and the remaining 124 were highly susceptible. The cultivar CO 6 and the accession NBPGR 1195-B did not show any visible symptoms and the virus could not be detected by back tests. With some of these newly found sources of resistance to CAMV, it may now be possible to incorporate this resistance genes from these species into cowpea varieties that have desirable horticultural characters through wide crossing or other innovative approaches in biotechnology.

## 5.11. *IN VITRO* CALLUS CULTURE AND REGENERATION OF COWPEA

Seeds of leguminous crops in general, and grain legumes in particular, carry viruses internally. Most of these viruses are transmitted either mechanically or through insect vectors or both. Spread of the virus within a growing crop usually results in extensive seed quality and yield losses (Kartha *et al*., 1981).

*In vitro* plant cell and tissue culture techniques are powerful tools for the development of new cultivars.

During the present investigations it was found that various explants tested responded differently on different media composition. 2,4-D was found to be the most suitable growth regulator for the induction of highly embryogenic callus in leaf and hypocotyl explants. Regeneration from the callus has not been observed in any of the media combination tried.

Direct regeneration from the apical portion of the stem apex was observed by many workers in *V.unguiculata* (Kartha *et al*.,1981; Cheema and Bawa, 1992; Rathor *et al*., 1994). Earlier Bajaj and Dhanju (1979) had reported the regeneration of plants from the apical meristem tips of different legumes. Singh *et al*. (1980) also got similar results using macerated shoot tips of *V.radiata* var. *aureus*. More studies have to be undertaken to standardize a media for regeneration of cowpea from callus cultures to develop new cultivars resistant to CAMV through somaclonal variation.

# *SUMMARY*

# CHAPTER 6

## SUMMARY

6.1. The virus causing mosaic disease of cowpea induced typical mosaic mottling with dark green and light green patches on leaves. In some varieties, infected plants exhibited dark green vein banding, leaf distortion blistering and stunting. The adult plants exhibited extensive necrotic streaks on the stem, leading to drying of the entire plant.

6.2. The virus could be transmitted through mechanical means and by *A.craccivora*, *A.gossypii* and *M.persicae*. It was also transmitted through seeds. However, *B.tabaci* and *C.trifurcata* failed to transmit the virus.

6.3. The data on virus-vector relationship showed that the virus could be efficiently transmitted by a short acquisition period of 5 min and could be transmitted with an inoculation feeding of one min. Influence of starvation before acquisition and inoculation proved that preacquisition starvation for 1 h produced maximum infection but preinoculation starvation decreased the per cent infection. Even a single aphid could transmit the disease. The viruliferous nature of the vector was lost after 2 h.

6.4. Anatomical studies revealed that there was severe curling of leaf primordia and the apical meristem tip was poorly developed in plants infected with the virus. The CS of infected leaves also showed heavy proliferation of cells.

6.5. Host range studies showed that the virus was restricted to four families viz., Amaranthaceae, Chenopodiaceae, Labiatae and Leguminosae.

6.6. Serological studies based on DAC-ELISA indicated that isolate from Coimbatore showed high reactivity with polyclonal and monoclonal antibodies specific to CAMV. They showed weak reaction with monoclonals specific to BICMV. NCM-ELISA technique also was able to detect the antigen even at 1:1000 dilution and in body fluid of single viruliferous aphid vector.

6.7. Electron microscopic studies revealed that the virus particles were flexuous, filmentous and were approximately 750 nm long.

6.8. Immunosorbent electron microscopy of CAMV also showed the filamentous nature of the particle and the virus could be efficiently trapped using polyclonal antisera specific to CAMV and potyvirus group antisera.

6.9. The physiology and biochemistry of virus infected plants revealed that there was significant reduction in chlorophyll content in inoculated, susceptible varieties. The level of carbohydrate content was significantly lower in susceptible varieties when inoculated with CAMV. There was an increase in total soluble protein content in infected plants compared to healthy control plants.

6.10. The SDS-PAGE analysis of seed protein revealed that there was significant difference in protein profile of infected and healthy seed samples. The protein band at 18.0 kDa was found to be more denser in diseased samples. Three novel proteins (PR-proteins) with molecular weight 34, 31 and 23 kDa were detected in inoculated cowpea plants treated with AVP.

6.11. The isozyme analysis performed to explain the nature of disease resistance indicated that there was significant variation in isoPOs in resistant and susceptible cultivars.

6.12. Inoculation with CAMV increased the RNA content in the susceptible C 152 and CO 4 varieties. The DNA content was low in inoculated susceptible cultivars, when compared to uninoculated control.

6.13. There was a progressive increase in peroxidase activity in inoculated susceptible variety CO 4 upto 5-day after treatment, then they showed a decline. The enzyme activity was enhanced on treatment with BSLE and on inoculation with CAMV after treatment with BSLE. The PPO activity also showed the similar trend. The susceptible varieties on inoculation showed a significant increase in PAL activity upto 10 days after treatment and then it showed a decline. The PAL activity was also increased on BSLE treatment and on inoculation after BSLE treatment. The chitinase activity also exhibited the same pattern of increase in plants which received the treatments. The level of all enzymes analysed in the resistant cultivar CO 6 maintained a higher level in treated and untreated control when compared to susceptible cultivars.

6.14. The total phenol content increased significantly in CAMV inoculated susceptible cultivars. The induction of phenol content was more at 3 days after inoculation and showed a decline from $4^{th}$ day after inoculation. The total phenol content was consistently more throughout the experimental period in leaves of resistant variety. The OD-phenol content also maintained the same trend as that of total phenol content.

6.15. Out of 20 virus inhibitory chemical tested four chemicals viz., barium chloride, thiouracil, ammonium molybdate and copper sulphate gave maximum inhibition of CAMV infection.

6.16. Among the 25 plant species tested *C.annum* showed complete inhibition of CAMV. *B.rubra*, *C.bonplandianum*, *I.carnea*, *M.jalapa*, *B.spectabilis*, *D.stramonium*, *P.chilensis*, *B.diffusa*, *P.pinnata*, *T.portulacastrum* and *L.leucocephala* showed more than 90 per cent inhibition when compared to control.

6.17. Plant extracts were also effective when applied by mixing with virus inoculum. Virus inoculum mixed with leaf extracts of *B.spectabilis*, *C.annum*, *C.bonplandianum* and *M.jalapa* gave cent per cent inhibition.

6.18 The protein fractions from MJLE, BSLE, ICLE and PCLE were separated using ammonium sulphate precipitation and they were highly effective in reducing the CAMV infection.

6.19 Treatments of cowpea seedlings with partially purified *Bougainvillea* antiviral protein (BAP) increased the peroxidase, polyphenoloxidase and phenylalanine ammonia-lyase activities.

6.20. Efficacy of four selected leaf extracts, MJLE, BSLE, ICLE and PCLE showing antiviral properties, two nonedible oils (neem and mahua oils) and a systemic insecticide (monocrotophos) were used for the management of CAMV under field conditions. Among the leaf extracts tried MJLE and BSLE showed

maximum inhibition of the disease, which was on par with mahua oil and monocrotophos. Neem oil was superior to all other treatments at 90 DAS.

6.21. Glasshouse trials using foliar sprays of leaf extracts and insecticides showed that maximum inhibition was found in plants treated with monocrotophos. Among the leaf extracts, MJLE was superior and showed maximum inhibition of (87.5 per cent) CAMV.

6.22. In the present investigation out of 332 cowpea genotypes screened for resistance to CAMV, 40 showed resistant reaction. Seventy eight were tolerant, 90 were susceptible and the remaining 124 were highly susceptible. The cultivar CO 6 and the accession NBPGR-1195-B were found to be highly resistant to CAMV.

6.23. *In vitro* callus culture and regeneration of cowpea showed that MS medium supplemented with 2,4-D (2.0 mg $l^{-1}$) + Kn (1.0 mg $l^{-1}$) gave maximum callusing of 86.67 per cent with leaf explant and MS + 2,4-D (2.5 mg $l^{-1}$) + Kn (1.0 mg $l^{-1}$) gave 85.0 per cent callusing with hypocotyl explant. The callus were highly granular and creamy white in colour. There was no regeneration from the callus derived from leaf and hypocotyl explants.

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