BIOCHEMICAL BASIS OF RESISTANCE AGAINST BLACKEYE COWPEA MOSAIC VIRUS IN COWPEA (*Vigna unguiculata* (L.) Walp.)

BY

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THESIS

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2001

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I hereby declare that this thesis entitled "**Biochemical basis of resistance against blackeye cowpea mosaic virus in cowpea (*Vigna unguiculata* (L.) Walp.)**" is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar title, of any other university or society.

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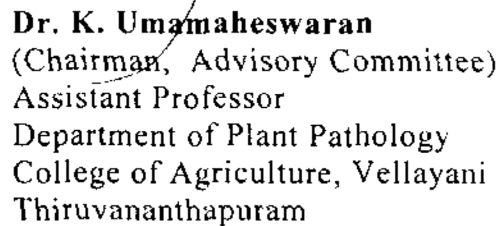

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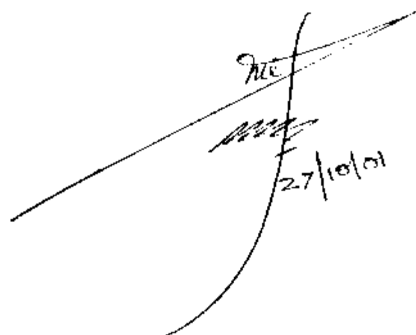

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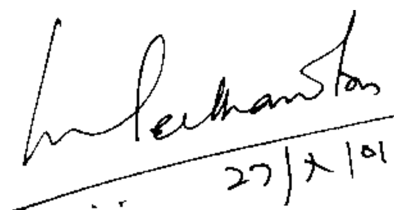

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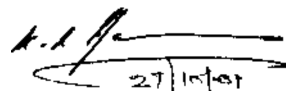

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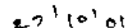
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Introduction

1. INTRODUCTION

Cowpea (*Vigna unguiculata* (L.) Walp.) is one of the most important high protein food in many tropical and subtropical countries. Among legumes, cowpea is one of the most important vegetable crops grown during rainy and summer seasons. It produces seeds, pods and leaves (all edible by human or livestock) with unusually high protein levels. The cowpea has important potential for small-scale farmers in our state. It is cultivated largely for its young tender pods and grains. The young leaves and shoots may also be used as a leafy vegetable.

Many viruses are known to infect cowpea and cause serious losses in many countries. Virus diseases are considered to be a major limiting factor affecting the production of cowpeas. Several viruses infect cowpea and many of them are seed transmitted. The most important seed-borne virus, the blackeye cowpea mosaic virus of cowpea in Kerala is aphid transmitted filamentous virus approximately 750 nm long (Radhika and Umamaheswaran, 2000). It was first isolated in Florida by Anderson (1955), who designated it as "blackeye cowpea mosaic virus" (BlCMV).

The research being reported here was undertaken to locate sources of heritable resistance to the virus and to study the biochemical basis of disease resistance as well as immunodetection of BlCMV in plant sample for the early diagnosis of the disease.

Review of Literature

2. REVIEW OF LITERATURE

2.1 Varietal screening

Ladipo and Allen (1979) screened cowpea germplasm and categorized 52 lines as immune, six lines as tolerant and all the remaining lines as susceptible to a Nigerian isolate of cowpea aphid borne mosaic virus (CABMV). Ramaiah (1978) identified that the culture MS 9804 was found to be resistant to CABMV, when 234 germplasm were screened using a new method based on disease indices. This was used to transfer its resistance to susceptible cultivars Co1 and Co2. Mali *et al.* (1981) observed the immunity of cowpea variety C-228 against cowpea aphid-borne mosaic virus. Walker *et al.* (1981) reported that a single gene *blc* control resistance to blackeye cowpea mosaic virus (BlCMV) in progeny from a cross between southern pea cultivars "Worth more" (homozygous resistant) and California Blackeye No:5 (homozygous susceptible). All F_1 plants and backcrosses to the susceptible parent were susceptible indicating that resistance was recessive to susceptibility. Five resistant lines were identified by Taiwo and Gonsalves (1982) from among 58 cowpea cultivars against BlCMV.

Provvidenti *et al.* (1983) reported that in populations from crosses of resistant and susceptible plants of the bean (*Phaseolus vulgaris*) cultivar Black Turtle Soup, resistant to blackeye cowpea mosaic virus (BlCMV) and to CABMV was conferred independently by single dominant factors that appeared to be closely linked to resistance.

Atiri and Thottappilly (1984) compared mechanical inoculation and aphid inoculation as modes for screening cowpeas for resistance against cowpea aphid borne mosaic virus (CABMV) and proved mechanical inoculation, to be a better method of CABMV transmission in aphid susceptible lines. Collins *et al.* (1985) inoculated 16 cowpea cultivars with major cowpea viruses and rated their susceptibility and reported that Brown Crowder, Magnolia Blackeye, Mississippi Silver Magnolia purple and Worthmore had promising levels of tolerance to BlCMV. Lima *et al.* (1986) screened 248 cultivars for resistance to cowpea severe mosaic and CABMV. They were mechanically inoculated and classified based on symptomatology and serology as immune, highly resistant, resistant, susceptible or highly susceptible. Sreelakha (1987) screened ten lines of cowpea varieties of which the variety C-152 was found to be highly susceptible and the variety CG-104 was tolerant to the disease. She also reported that lines TVu-9836, TVu-9914, TVu-9929, TVu-9930, TVu-9944 were resistant to CABMV.

Cowpea varieties IT 82D-889, IT 83S-818, IT 83D-442 and IT85 F-867-S were resistant to cowpea mosaic como virus (CPMV), CABMV, cowpea golden mosaic gemini virus (CGMV), cucumber mosaic cucumovirus and southern bean mosaic virus (SBMV) (Singh *et al.*, 1987; Thottappilly and Rossel, 1985; Singh *et al.*, 1997). Mali *et al.* (1988) screened 60 cultivars for the presence of BlCMV and CABMV and identified BlCMV from 19 cultivars and CABMV from 7, based on transmission and serological tests. Quindere and Barreto (1988) evaluated 81 genotypes of cowpea and classified seven as resistant to cowpea severe mosaic and CABMV. They reported that the variety CNC XII-OD was found to be resistant to the virus.

Ndiaye *et al.* (1993) evaluated 35 genotypes and found TVu-401, TVu-498 P_2, TVu-1000, TVu-1016-1, TVu-1582 were resistant to all isolates of poty viruses affecting cowpea.

Sudhakumari (1993) screened 59 varieties and identified V-317 and V-276 were resistant to CABMV. Kannan and Doraiswamy (1994) screened 50 cowpea varieties against BlCMV under glasshouse condition. Sap from eight virus infected weed species were also used for their inoculation studies. Among the varieties tested, 30 were free from infection. Miller and Scheuring (1994) released Texas pink eye purple Hull cowpea belonging to *Vigna unguiculata*, which was found to be immune to two American isolates of CABMV. Ponte *et al.* (1994) reported the reaction of cowpea cultivar 'Pampo' to three viruses and showed that the variety was highly susceptible to cowpea severe mosaic and CABMV. Resistance was observed against cucumber mosaic comovirus also.

Bashir *et al.* (1995) screened fifty cowpea genotypes for resistance to seed-borne isolate of BlCMV by mechanical inoculation. They found that ten genotypes (IT86F 2089-5, IT86 D-880, IT86 F 2062-5, IT92 KD-266-2-1, IT90 K-284-2, IT90 K-76, IT86 D-1010, IT87 D-611-3, TVu-7676 and PAK 45443) were resistant to BlCMV.

Bashir and Hampton (1996) tested 51 lines of cowpea by mechanical inoculation against seven diverse isolates of BlCMV and found that five genotypes IT 8082049, Big Boy, Corona, Serido and Tennessee Cream were immune to all the seven isolates tested. Three genotypes TVu-2657, TVu-2740 and TVu-3435 were immune to six isolates of the virus tested.

Kline and Anderson (1997b) released UARK-M2, progeny of single plant selections of symptomless *V. unguiculata* cv. Coronet from the commercial fields where high incidence of BlCMV was noticed. The variety was high yielding and resistant.

Gumedzoe *et al.* (1998) evaluated cowpea germplasm accession and breeding lines using BlCMV and CABMV isolates and found that germplasm accessions TVu 401, TVu 1453 and TVu 1948 and breeding lines IT 82 D-885, IT 82 D-889 and IT 82 E-60 possess resistant genes to all six poty virus isolates and recommended that they could be used for breeding programme at IITA to develop new cowpea varieties. Van *et al.* (2000) analysed 14 cowpea lines under glass house condition for susceptibility to infection by three isolates of BlCMV and 10 isolates of CABMV.

2.2 Biochemical changes of host-pathogen interaction

2.2.1 Carbohydrate

Khatri and Chenulu (1969) reported that reducing sugar content was not appreciably affected by cowpea mosaic virus in resistant and susceptible cowpea cultivars. Ramaiah (1978) found that there was decreased synthesis of total carbohydrates in infected leaves of susceptible cowpea. He also observed that the trifoliate leaves showed reduction in the level of carbohydrates commencing from the 10th day after inoculation. Singh and Singh (1984) observed that the virus infection decreased total sugar and starch in cowpea cultivars infected with southern bean mosaic virus and CPMV. Johri and Pandhi (1985) reported that the carbohydrate level declined with severity of disease symptoms in case of yellow vein mosaic of okra.

Sastry and Nayudu (1988) recorded a higher quantity of carbohydrate in hypersensitive cowpea cultivars infected with tobacco ring spot NEPO virus and suggested that the infected area acts as a metabolic sink. Mayoral *et al.* (1989) reported that carbohydrate level was much reduced in infected leaf tissues. Yellow vein mosaic virus infection reduced the chemical constituents of bhindi like reducing sugar, total sugar etc. (Sarma *et al.*, 1995).

Dantre *et al.* (1996) reported that in the case of yellow mosaic virus infecting soybean, reducing sugar, non-reducing sugar and total sugar decreased in infected leaves. Thind *et al.* (1996) reported that the amount of reducing sugars, non-reducing sugars, total sugars and starch decreased in plants infected with yellow mosaic virus when compared to healthy control in case of yellow mosaic virus infecting mung plant. Umamaheswaran (1996) found that the level of carbohydrate was significantly lower in susceptible varieties of cowpea when inoculated with CABMV.

Bhagat and Yadav (1997) reported that healthy leaves of susceptible and highly susceptible cultivars showed higher content of reducing, non-reducing and total sugar than resistant one in the case of bhindi yellow vein mosaic virus infected plants. It was also reported that increased sugar content in inoculated leaves of bhindi was due to their accumulation, as a result of the disruption of normal phloem transport. Mali *et al.* (2000) reported that in the case of yellow mosaic virus infecting moth bean, the reduction in content of total soluble carbohydrate was more in susceptible than in resistant genotype.

2.2.2 Chlorophyll

There was significant reduction in chlorophyll content in virus infected susceptible variety Co-2 (Ramaiah, 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 the infected plants. There was pronounced reduction in chlorophyll content in plants infected with cowpea mosaic virus but the content of the carotenoid was unchanged. The total chlorophyll, chlorophyll a and b increased upto 60 days after inoculation and then declined (Rao and Shukla, 1989). They suggested that the yield loss due to virus infection was principally due to a reduced rate of photosynthesis.

Kaur *et al.* (1991) found that infection of yellow mosaic virus in soybean cultivars reduced the chlorophyll content. Wani *et al.* (1991) recorded reduction in total chlorophyll in sorghum leaves infected with maize mosaic virus. Shukla *et al.* (1992) reported that there was a decline in chlorophyll content due to sugarcane mosaic virus and ratoon stunting disease infection in some sugar cane varieties. Sarma *et al.* (1995) reported the reduction of total chlorophyll and chlorophyll b in case of yellow vein mosaic virus infecting bhindi. Biochemical changes induced by yellow vein mosaic virus in leaves of soybean cultivars was studied by Dantre *et al.* (1996) and reported that the reduction of total chlorophyll, chlorophyll a and chlorophyll b were due to infection by the virus. Thind *et al.* (1996) reported the reduction in total chlorophyll in yellow mosaic affected leaves of mung plants when

compared with healthy control. Mali *et al.* (2000) reported a reduction in content of chlorophyll a, b and carotenoids in susceptible than in resistant genotype following yellow mosaic virus infection in moth bean (*Vigna aconitifolia*). Radhika and Umamaheswaran (2000) reported a higher chlorophyll content in resistant variety when compared to susceptible variety in case of cowpea infected with BlCMV.

2.2.3 Phenol

Ramaiah (1978) found that there was no difference in phenol content between healthy and inoculated leaves of MS 9804 and CO-1. He found that in variety CO-2 the inoculated leaves had higher content of phenol than that of healthy leaves at 40 days after inoculation with CABMV. Ando *et al.* (1984) reported that fungitoxic phenolic compounds were released from cucumber mosaic virus infected cowpea protoplast. Sharma *et al.* (1984) studied the effect of virus and fungus infection in musk melon and showed an increasing trend on the enzyme activity and phenol content as compared to healthy control irrespective of the nature of infection. Rathi *et al.* (1986) assayed total phenol and other biochemical parameters in pigeonpea cultivars resistant and susceptible to sterility mosaic virus and reported that there was not much differences between varieties with respect to total phenol content. Ahmed *et al.* (1992) reported that the total phenol, orthodihydroxy phenol and flavanols were found high in virus free resistant varieties of okra. He also reported that after inoculation with yellow vein mosaic virus, total phenols, orthodihydroxy phenols and flavanols decreased in resistant lines. Zaidi *et al.* (1992) suggested that there was a correlation between elevated levels of phenolics with disease resistance in the case of carnation etched ring virus

affecting carnation. Kato *et al.* (1993) extracted and characterized two phenolic compounds from cowpea leaves infected with cucumber mosaic virus. Sohal and Bajaj (1993) reported increase in total phenols in both resistant and susceptible varieties of mung bean infected with yellow mosaic virus. Sarma *et al.* (1995) reported the increase in total phenol as a result of infection on bhindi plants by yellow vein mosaic virus. Dantre *et al.* (1996) reported that the total phenol content increased in leaves of soybean plant infected with yellow mosaic virus. Sutha *et al.* (1997) found that both total phenol and ortho-dihydroxy phenol increased in tomato spotted wilt virus infected plants. Mali *et al.* (2000) reported that ortho-dihydroxy phenol was higher in healthy leaves than diseased leaves in case of yellow mosaic virus affecting moth bean.

2.2.4 Protein

Padma *et al.* (1976) reported that cowpea mosaic virus infected seeds contained a higher percentage of proteins, than healthy seeds. Singh *et al.* (1978) found that southern bean mosaic virus infection resulted in higher total nitrogen, total protein, nitrate and nitrite nitrogen than in healthy leaves of cowpea. Singh and Singh (1981) while investigating the changes in nitrogenous constituents of cowpea pods due to cowpea mosaic virus, found that there was an increase in total nitrogen, protein and nitrate nitrogen. Johri and Pandhi (1985) while studying the effect of yellow vein mosaic on the physiology of okra, reported that the total protein contents declined in diseased tissues while its insoluble fraction increased in diseased tissues as against soluble fraction which was lower in diseased tissue. Ahmed *et al.* (1992) reported that total proteins and soluble proteins were found high in

virus free resistant varieties. Mali *et al.* (2000) reported that free amino acids and soluble protein content increased with increasing levels of yellow mosaic virus infection in susceptible variety of moth bean.

2.2.5 Defence related enzymes

Khatri and Chenulu (1970) studied the changes in the peroxidase enzyme activity in leaves of resistant and susceptible cowpea varieties and observed that peroxidase activity increased in both resistant and susceptible varieties but was higher in susceptible variety. Batra and Kuhn (1975) found that when primary leaves of hypersensitive soybean plants were infected with cowpea chlorotic mottle virus, the enzymes polyphenol oxidase and peroxidase increased 2-3 times. They found that the increase was concomitant with the development of acquired resistance.

Wagih and Coutts (1982) reported that tobacco necrosis virus infected cowpea and cucumber showed alterations in soluble protein accompanied by an increase in the amount of extractable peroxidase and polyphenol oxidase activity. Sharma *et al.* (1984) while studying the effect of virus and fungus infection in musk melon showed an increasing trend of enzymic activity when compared to healthy control. Rathi *et al.* (1986) assayed peroxidase, polyphenol oxidase and isozyme of peroxidase in pigeonpea cultivars resistant and susceptible to sterility mosaic disease and noted less difference between two varieties with respect to peroxidase and polyphenol oxidase activity, which increased in susceptible cultivars following infection. Resistance was characterized by the presence of specific isoperoxidase and protein.

Zaidi *et al.* (1992) reported the changes in phenolic content and phenyl alanine ammonialyase in response to infection by carnation etched ring virus. The results strongly suggest the existence of a correlation between the elevated levels of phenolics and phenyl alanine ammonialyase with disease resistance. Ahmed *et al.* (1992) found that enzyme peroxidase and polyphenol oxidase showed no significant difference in virus free susceptible and resistant ones while studying biochemical basis of resistance to yellow vein mosaic virus in okra. Sohal and Bajaj (1993) reported increase in polyphenol oxidase activity in resistant variety of mung bean infected with yellow mosaic virus.

Umamaheswaran (1996) reported that there was progressive increase in peroxidase, polyphenol oxidase and phenyl alanine ammonialyase activity in inoculated and susceptible varieties of cowpea. Mali *et al.* (2000) reported that the activity of catalase, peroxidase and nitrate reductase enzymes decreasing with increasing intensity of disease in case of yellow mosaic disease of moth bean (*Vigna aconitifolia*). Radhika and Umamaheswaran (2000) reported higher activity of peroxidase polyphenol oxidase and phenyl alanine ammonialyase in resistant variety when compared to susceptible variety of cowpea infected with BlCMV.

2.2.6 Electrophoresis of soluble proteins

Lima *et al.* (1979) reported that sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) dissociated inclusions and virions revealed that inclusions consisted of a single protein estimated to have a molecular weight of 70,000 Da whereas freshly purified BlCMV consisted of a main protein

component with molecular weight of 34,000 Da and two smaller protein with molecular weight of 29,000 and 27,000 Da. Rottier *et al.* (1980) while investigating protein sythesis 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, all other virus – related protein appeared to be synthesized at increasing rates.

Taiwo and Gonasalves (1982) purified six isolates of blackeye cowpea mosaic virus and the coat protein used in antiserum production was analysed by SDS-PAGE. They reported that one purified preparation of BlCMV – Fla2 used for immunization had three bands. Molecular weight estimated for these bands in 7.5 per cent gels were 34,000 – 35,000, 31,000 and 29,000 Da for top, middle and bottom bands.

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 novel host proteins as α and β fractions in cowpea leaves, induced during virus – elicited necrosis. Gonsalves *et al.* (1986) reported the presence of three protein species with molecular weights of about 37,500, 32,200 and 29,000 Da when purified preparations of cardamom mosaic virus were denatured with sodium dodecyl sulfate (SDS) and analysed by electrophoresis in polyacrylamide gels.

Sreenivasulu and Demski (1992) dissociated protein coat of four purified peanut viruses which were analysed by SDS-polyacrylamide slab gel electrophoresis. They reported that the dissociated coat proteins of the four viruses contained one major band with electrophoretic mobility corresponding to a protein with a molecular weight of 34,000 Da. In addition to the major band at least one minor band migrating faster than the major one was detected.

Thottappilly *et al.* (1993) determined the molecular weight of viral coat protein of a cowpea chlorotic mottle virus isolate from Nigeria using SDS-PAGE and reported that CCMV virions had a single coat protein with an apparent molecular weight of 20,000 Da as determined by SDS-PAGE in 12.5 per cent gel. Yoshikawa *et al.* (1993) reported four PR proteins in cowpea leaves infected with cucumber mosaic virus and designated Cp PR-1, Cp PR-2, Cp PR-3 and Cp PR-4 in the order of faster migration. They also detected Cp PRs production at the time of necrotic local lesion appearance and increased as the lesion matured. Raj *et al.* (1994) reported a virus causing mottling, ringspots, green mosaic and stunting of growth in *Dimorphotheca aurantiaca.* Analysis of the virion protein by SDS-PAGE showed a single virus protein of 55 kDa. Satyanarayana *et al.* (1994) identified a strain of peanut chlorotic streak virus causing chlorotic vein banding disease of groundnut in India and reported that in SDS-PAGE the virus coat protein resolved into two major polypeptides of 76 kDa and 36 kDa.

Jensen (1996) reported a new disease of maize and wheat in the highplains. The nucleoprotein of pathogen was isolated from infected tissue

and analysed by SDS-PAGE revealing the coat protein of wheat streak mosaic virus (WSMV) and a unique, approximately 32 kDa protein. Montana *et al.* (1996) reported the presence of capsid protein of about 45 kDa, while serological characterization of WSMV isolate. Several bands below and sometimes above 45 kDa were also observed from several of the isolates. Ribeiro *et al.* (1996) estimated the molecular mass of eggplant mosaic virus by SDS-PAGE and reported that EMV-T capsid was resolved as a single polypeptide with an estimated molecular mass of 22,000 Da. Huguenot *et al.* (1997) conducted a study of the capsid proteins of different poty viruses infecting legumes using specific monoclonal antibodies on immunoblots of crude extracts from infected plants revealed that CABMV and BICMV had coat protein of M_r values of 32 kDa and 35 kDa respectively.

2.2.7 Electrophoretic analysis of isozymes

Brown *et al.* (1978) found that the isozyme polymorphism was a useful indicator of diversity of genotypes. Isozyme analysis is a powerful tool for estimating genetic variability, identifying cultivars and germplasm accessions (Asiedu, 1992). Umamaheswaran (1996) indicated that there was significant variation in peroxidase isozyme in resistant and susceptible cultivars. Sonnate *et al.* (1997) evaluated different species of *Vigna* and found that there was very low similarity among species in the isozymes analysed.

2.2.8 Qualitative determination of free amino acids

Roberts *et al.* (1952) recorded the accumulation of serine, threonine and proline in turnip infected with turnip yellow mosaic virus.

Miczynski (1959) reported that in tobacco variety White Burley inoculated with a non-necrotic ring spot strain of potato virus X, there was an increase in the concentration of amino acids even before the expression of disease symptoms. But during the rapid spread of the virus, he found a lesser concentration in the diseased leaves than in healthy ones. Once the disease was well established the older leaves showed a general tendency towards increase in concentration over that of healthy plant. He observed a steady increase in aspartic acid and leucine and a decrease in glutamic acid in the diseased leaves. Diener (1960) found that proline was present throughout the season in leaves of peach infected with western X virus as against a reduction during summer in healthy leaves.

Bozarth and Diener (1963) noted an increase in the concentration of glutamic acid, serine, aspargine, amino-butyric acid and proline in tobacco plants infected with potato virus X and Y. John (1963a,b) recorded an increase in free amino acids in *Dolichos lablab* infected with DEMV and TMV. The maximum level was reached between 10-20 DAI in *Dolichos lablab* and eight days in tobacco. The increase was noticed in the case of alanine, lysine, proline, tryptophane and *tyrosine* in TMV infected tobacco.

Narayanaswamy and Ramakrishnan (1966) noted a tendency for increase in the free amino acid contents of pigeon pea infected with sterility mosaic virus. According to them there was diurnal variation in certain amino acids. The increase in concentration of arginine, alanine, aspartic acid and asparagines, they noted, was proportional to the severity of the disease in pigeon pea leaves. Daniel (1968) detected valine in the leaves of mosaic

affected amaranthus in addition to glutamine, arginine and alanine in healthy leaves. Balakrishnan Nair (1969) reported that amino acids in diseased banana plants infected with banana bunchy top mosaic virus were higher than that in healthy.

2.3 Immunodetection

Fischer and Lockhart (1976) performed Outcherlony test to prove that an isolate of CABMV was not related to bean common mosaic virus. Double immuno diffusion test was conducted to prove the relationship of BlCMV with other potyviruses and found that BlCMV was serologically related to but distinct from other potyviruses tested (Lima *et al.*, 1979). Taiwo and Gonsalves (1982) studied the relationship between two isolates of BlCMV and four isolates of CABMV using double diffusion methods and found that the antisera of isolates of CABMV group did not react with isolates of BlCMV group. Dijkstra *et al.* (1987) used ELISA to differentiate BlCMV and CABMV isolates from yard long bean. Zhao *et al.* (1991) conducted ELISA to prove that BlCMV from Alyce-Clover is related to BlCMV isolate of cowpea form Florida and South Carolina, but was less related to CABMV, peanut poty virus and nine other poty virus.

Huguenot *et al.* (1992) conducted ELISA and confirmed that BlCMV and CABMV were two different potyviruses. Gillaspie *et al.* (1993) studied the seed transmission of blackeye cowpea mosaic potyvirus in cowpea using DAS-ELISA. Gumodzoe (1993) reported the use of immunosorbent assays for the identification of virus diseases of cowpea in Togo. Ndiaye *et al.* (1993) used direct antigen coating (DAC) and double antibody sandwich

(DAS) ELISA for the detection of seven seed-borne viruses in cowpea seeds. Chang *et al.* (1994) used ELISA for detecting BlCMV and infected plant was rouged out for disease control. Bashir and Hampton (1995) conducted DAS and DAC ELISA to identity CABMV from infected cowpea plants. Anderson *et al.* (1996) used ELISA for the evaluation of cowpea lines for resistance against BlCMV. Konate and Neya (1996) proved the reliability of ELISA technique for selecting CABMV free stock of cowpea seeds. Wall and Kimmons (1996) conducted ELISA for detection of BlCMV in yard-long bean in Mariana Islands.

Kline and Anderson (1997) detected the presence of CABMV from cowpea grown commercially in United States using ELISA. Shoyinka *et al.* (1997) used ELISA to detect viruses in 649 cowpea leaf samples. Survey was conducted for six viruses including BlCMV and CABMV. Gumedzoe *et al.* (1998) used Serological tests (ACP and DAS-ELISA) with biotin-labelled monoclonal and polyclonal antibodies to identify BlCMV and CABMV serotypes of both poty virus. Radhika and Umamaheswaran (2000) identified that the virus causing mosaic disease in cowpea as black eye cowpea mosaic virus using DAS and DAC-ELISA.

Materials and Methods

3. MATERIALS AND METHODS

3.1 Screening for disease resistance

Sixty six germplasm collections of cowpea were screened for blackeye cowpea mosaic virus. Local cultivars from Thiruvananthapuram district and other available cultivars from Regional Agricultural Research Station (RARS), Pattambi, National Bureau of Plant Genetic Resource (NBPGR), New Delhi and from farmers field were collected and screened for virus resistance based on 0-5 scale proposed by Rajamony *et al.* (1990).

Seeds collected were sown in pots, filled with potting mixture containing soil, sand and cowdung in the ratio of 1 : 1 : 1. The seedlings in the primary leaf stage were mechanically inoculated with the sap extracted from diseased leaves. 0.01 M phosphate buffer, pH 7.0 was used for all inoculation studies and 600 mesh carborundum powder was used as abrasive. Sap was extracted from young tender leaves of cowpea plants showing severe mosaic symptom maintained in insect proof glass house. One part of the leaf tissue was homogenized with one part of cold buffer (1 : 1 w/v) in a mortar and pestle under cold condition. The homogenate was strained through a double layer of muslin cloth and maintained under cold condition till it was inoculated to test plants. Prior to inoculation, primary leaves of the test plants were uniformly dusted with carborundum powder. Thereafter the inoculum was gently rubbed on the upper surface of fully opened primary leaves with the fore finger. The surface of the inoculated leaf was rinsed off after 10 minutes using distilled water. The plants were then kept under observation for the development of symptom.

The varieties were screened by scoring the symptoms as proposed by Rajamony *et al.* (1990). Screening was carried out in an insect proof glass house and observations were recorded 30 days after inoculation using the following scale.

0 – No symptom

1 – Slight vein clearing on young leaves

2 – Leaves with light and dark green patches

3 – Blisters and mottling on the leaves

4. – Severe mottling and distortion of leaves

5 – Stunting of the plants with negligible or no flowering and fruiting

Based on the rating, Vulnerability index (VI) was calculated using the following equation.

$$VI = \frac{(0n_0 + 1n_1 + 2n_2 + 3n_3 + 4n_4 + 5n_5)}{n_t (n_c - 1)} \times 100$$

VI = Vulnerability index

$n_0, n_1 \ldots\ldots n_5$ = number of plants in the category 0, 1, 2, 3, 4, 5.

n_t – total number of plants

n_c – total number of categories.

3.1.1 Breeding for resistance

Sharika, Malika (varieties released from Kerala Agricultural University) and a local accession, Pallichal local belonging to *Vigna unguiculata* sub sp. *sesquipedalis* (vegetable type) commonly cultivated by farmers were found

less tolerant to BlCMV and other viruses of cowpea. These varieties were crossed with the resistant variety CO-6, *V. unguiculata* sub sp. *unguiculata* a grain type obtained from Tamil Nadu Agricultural University. The following cross combinations were made to develop resistant lines against BlCMV.

Sharika x Co-6

Co-6 x Sharika

Malika x Co-6

Co-6 x Malika

Pallichal local x Co-6

Co-6 x Pallichal local

The hybrid seeds produced from the above cross combinations were collected for evaluation.

3.2 Biochemical changes of host-pathogen interaction

Biochemical analysis of resistant and susceptible varieties were carried out. Co-6 was selected as resistant variety and Sharika as susceptible variety. Seeds of both varieties were sown in insect proof glass house and mechanically inoculated at primary leaf stage. Samples were taken one day, five days, ten days and fifteen days after inoculation.

Biochemical analysis was conducted to estimate the changes in total carbohydrates, chlorophyll, phenol and protein. Analysis of defence related enzymes such as peroxidase, polyphenol oxidase and phenylalanine ammonialyase were also done. Protein profile study was done using SDS-PAGE and amino acid pattern was analysed using thin layer chromatography. Isozyme analysis of parents used for hybridization were also included in the present investigation using native PAGE (non-denaturating gel electrophoresis).

3.2.1 Estimation of total carbohydrate

Total carbohydrate content was estimated by Anthrone method (Hedge and Hofreiter, 1962). Samples of 100 mg each were weighed out and hydrolysed with five ml of 2.5 N hydrochloric acid (HCl) at 100^{0}C in a water bath. The hydrolysate was neutralized with solid sodium carbonate until the effervescence ceased. The volume was made up to 100 ml and centrifuged at 5000 rpm for 15 minutes. From the supernatant 0.5 ml aliquot was taken and made up to one ml by adding distilled water. To this four ml anthrone reagent was added and heated for eight minutes at 100^{0}C in a water bath. This was cooled rapidly and absorbance was measured at 630nm in a spectrophotometer (Systronics UV-VIS Spectrophotometer 118). Amount of carbohydrate present was calculated from the standard graph prepared using glucose and expressed in terms of milligrams of glucose equivalent per gram of leaf tissue on fresh weight basis.

3.2.2 Estimation of chlorophyll

Chlorophyll was estimated by the method described by Arnon (1949). One gram of leaf sample was finely cut and ground in a mortar with 20 ml of 80 per cent acetone. The homogenate was centrifuged at 5000 rpm for five minutes and the supernatant was transferred to a 100 ml volumetric flask. The above procedure was continued till the residue became colourless. The final volume was made upto 100 ml in a volumetric flask. Absorbance of the sample at 645 and 663 nm was read in a spectrophotometer (Systronics UV-VIS spectrophotometer 118) against the solvent blank (80 per cent acetone). The chlorophyll content was calculated using the following equations and expressed as milligram chlorophyll per gram tissue.

$$\text{Chlorophyll a} = 12.7 (A\ 663) - 2.69 (A\ 645) \times \frac{V}{1000 \times W}$$

$$\text{Chlorophyll b} = 22.9 (A\ 645) - 4.68 (A\ 663) \times \frac{V}{1000 \times W}$$

$$\text{Total Chlorophyll} = 20.2 (A\ 645) + 8.02 (A\ 663) \times \frac{V}{1000 \times W}$$

3.2.3 Estimation of phenol

The phenol content was estimated following the procedure described by Bray and Thorpe (1954). One gram leaf sample was ground in 10 ml of 80 per cent ethanol. The homogenate was, centrifuged at 10,000 rpm for 20 min. Supernatant was saved and residue was extracted with five times the volume of 80 per cent ethanol and centrifuged. The supernatant was saved and evaporated to dryness. The residue was dissolved in 5 ml distilled water. Folin-Ciocalteau reagent (0.5 ml) and two ml of 20 per cent sodium carbonate solution was added to each tube after three minutes. The reaction mixture was thoroughly mixed and kept in boiling water for one minute. This was cooled and the absorbance was measured at 650 nm against reagent blank. Standard curve was prepared using different concentrations of catechol and expressed in catechol equivalents as microgram per gram leaf tissue on fresh weight basis.

3.2.4 Estimation of protein

Total soluble protein content was estimated as per the procedure described by Bradford (1976). One gram of leaf sample was homogenized in 10 ml, 0.1 M Sodium acetate buffer (pH 4.7) and centrifuged at 5000 rpm for

15 minutes at 4^0C. The supernatant was saved for estimation of soluble protein. The reaction mixture consisted of 0.5 ml enzyme extract, 0.5 ml distilled water and 5 ml of diluted (5 times) dye solution (Coomassie brilliant blue G250). The absorbance was read at 595nm in a spectrophotometer (Systromics UV-VIS spectrophotometer 118) against reagent blank. Bovine serum albumin was used as the protein standard. The protein content was expressed as microgram albumin equivalent of soluble protein per gram on fresh weight basis.

3.2.5 Estimation of defense related enzymes

3.2.5.1 Estimation of peroxidase (PO)

Peroxidase activity was determined according to the procedure described by Srivastava (1987). Leaf sample of 200 mg was homogenized in one ml of 0.1 M sodium phosphate buffer (pH 6.5) to which a pinch of polyvinyl pyrrolidone (PVP) was added. The homogenization was done at 4^0C using a mortar and pestle. The homogenate was filtered through a muslin cloth and centrifuged at 5000 rpm for 15 minutes at 4^0C. The supernatant was used as the enzyme extract for the assay of PO activity.

The reaction mixture consisting of 1 ml 0.05 M pyrogallol and 50 μl of enzyme extract was taken in both reference and sample cuvettes, mixed and kept in a spectrophotometer (Systronics UV-VIS spectrophotometer 118) and the reading was adjusted to zero at 420 nm. The enzyme reaction was started by adding one ml of one per cent hydrogen peroxide (H_2O_2) into sample cuvettes and change in absorbance was measured at 30 seconds interval.

3.2.5.2 Estimation of 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 the estimation of peroxidase.

The reaction mixture contained one ml of 0.1 M sodium phosphate buffer (pH 6.5) and 50 μl of enzyme extract. The reaction was initiated after adding one ml of 0.01 M catechol. The observations were recorded in a spectrophotometer (Systronics UV-VIS spectrophotometer 118). The change in absorbance was recorded at 495nm and PPO activity was expressed as change in the absorbance of the reaction mixture per minute per gram on fresh weight basis.

3.2.5.3 Estimation of phenylalanine ammonialyase (PAL)

PAL activity was analysed based on the procedure described by Dickerson *et al.* (1984). The enzyme extract was prepared by homogenizing one gram leaf sample in five ml of 0.1 M sodium borate buffer (pH 8.8) containing a pinch of PVP using chilled mortar and pestle. The homogenate was centrifuged at 10000 rpm for 10 minutes at 4^0C. The supernatant was used for the assay of PAL activity. The reaction mixture contained three ml of 0.1 M sodium borate buffer (pH 8.8), 0.2 ml enzyme extract and 0.1 ml of 12 mM L-phenyl alanine prepared in the same buffer. The blank contained three ml of 0.1 M sodium borate buffer (pH 8.8) and 0.2 ml enzyme extract. The reaction mixture and blank was incubated at 40^0C for 30 minutes and reaction was stopped by adding 0.2 ml of 3N hydrochloric acid (HCl). The absorbance was read at 290 nm in a spectrophotometer (Systronics UV-VIS spectrophotometer 118).

PAL activity was expressed as nanomoles of cinnamic acid produced per minute per gram on fresh weight basis.

3.2.6 Electrophoretic analysis of proteins

Electrophoretic separation of soluble proteins of leaves were carried as per procedure described by Laemelli (1970). Leaf samples of susceptible (from healthy and diseased) and resistant plants were taken for analysis. One gram sample was homogenized in one ml of cold phosphate buffer (pH 7.0) at 4^0C. The homogenate was filtered through cheese cloth and centrifuged at 10,000 rpm for 20 min at 4^0C. The supernatent was taken and was precipitated by adding equal quantity of cold acetone.

The precipitated proteins were pelleted by centrifugation at 10,000 rpm for 15 minutes at 4^0C. The pellets were dissolved in sample buffer and vortexed. The vortexed samples were boiled at 95^0C for 4 min, in a water bath 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 following Bradford method.

Reagents

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 appromixately 50 ml of double distilled water. The pH was adjusted to 8.8 with 6 N HCl and made up the volume to 100 ml with double distilled water and stored at 4^0C.

c) Stacking gel buffer stock (0.5 M 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 6 N HCl and the volume was made upto 100 ml with double distilled water and stored at 4^0C.

d) Polymerising agents

Ammonium persulphate (APS) 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

Trisbase	6.0 g
Glycine	28.8 g
SDS	2.0 g
Double distilled water	2 l

f) Sample buffer (SDS-reducing buffer)

Double distilled water	2.6 ml
0.5 M Tris-HCl pH 6.8	1.0 ml
2 Mercaptoethanol	0.8 ml

Glycerol	1.6 ml
SDS 20 % (W/V)	1.6 ml
0.5 % Bromophenol blue	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 R250.

Procedure

Separating gel was first casted followed by 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
SDS 10 %	0.2 ml
Acrylamide stock	8.0 ml

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

10 per cent APS (freshly prepared)	0.10 ml
TEMED	0.01 ml

The separating gel was mixed well and poured immediately between glass plates and a layer of water was added above the polymerizing 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
SDS 10 %	0.1 ml
Acrylamide stock	1.3 ml

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

APS 10 %	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 100 V till the dye reached the separating gel. Then the voltage was increased to 200 V and continued till the dye reached the bottom of the gel. The gel was removed immediately after electrophoresis between the glass plates and incubated in the staining solution for over night with uniform shaking. Then the gel was transferred to the destaining solution. The protein appeared as bands and the gel was photographed after placing it on a transilluminator (Appligene Model White/UV TMW-20).

3.2.7 Electrophoretic analysis of isozymes

Isozyme analysis is a powerful technique for estimating genetic variability, identifying cultivars and germplasm accessions. Electrophoresis of protein extracts from plant tissues 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 present work was undertaken to study the enzyme alterations in primary leaves of parent lines of cowpea (used for hybridization) and to elucidate the polyphenol oxidase (PPO) isozyme profiles of genotypes, Sharika, Malika, Co-6 and Pallichal local selected during the course of screening.

Enzyme extraction and assay

Soluble and ionically bound enzymes were extracted by grinding five g of leaf tissue at 4^0C in 5 ml of 0.1 M sodium phosphate buffer (pH 7.2) with a mortar and pestle. The homogenate was incubated at 4^0C for one hour to complete the extraction and filtered through a double layer of cheese cloth. The filtrate was clarified by centrifugation at 10,000 rpm for 20 minutes. The resulting supernatant was used for isozyme analysis. Total protein content of the samples were determined by Bradford method.

Isozyme separation and staining

Discontinuous anionic polyacrylamide gel electrophoresis was conducted under non-dissociating conditions as previously described by Wagih and Coutts (1982). Proteins extracted by 0.1 M phosphate buffer (pH 7.2) were separated by gel electrophoresis in 7.5 per cent gel. The gel was prepared

using the stock solution prepared for protein gel electrophoresis with slight modification.

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

Following electrophoresis, the gel was immersed in a solution of 10 mM L-3,4 dihydroxy phenylalanine (L-DOPA) in 100 mM sodium

phosphate (pH 7.0) in a plastic tray kept in a shaker for 30 min. Zones of enzyme activity (PPO isozymes) were observed as grey black bands. The R_f values and relative intensities of the isozyme bands of PPO were also recorded.

3.2.8 Qualitative determination of free amino acids

Chromatography is a powerful technique to separate chemically closely related substances such as amino acids, sugars into individual components on the basis of their physiochemical properties. The compounds are separated on the basis of their partition coefficients between two immiscible phases.

Alcohol extraction of free amino acids

Five gram of leaf material was chopped into fine pieces and ground well after adding 2.5 ml of 80 per cent ethyl alcohol (2 : 1 w/v). The pulp was then transferred to a volumetric flask and ethyl alcohol was added and the volume was made upto 25 ml. The mixture was placed in a refrigerator for two hours for cold extraction. The material was then filtered under reduced pressure through Whatman No:42 filter paper. The residue in the filter paper was washed twice with alcohol and the filtrates were pooled. This was evaporated to dryness in vacuum. The residue was dissolved in 3 ml of 10 per cent iso-propyl alcohol and used for analysis.

The samples, healthy, diseased and standards were spotted on a precast TLC aluminium sheets 5 x 10 cm, silica gel 60 F_{254} from Merck Germany. The spots were dried using a hair drier.

The plates were placed in developing solvent n-butanol acetic acid water (4 : 1 : 1 v/v) in a 500 ml glass beaker. After allowing solvent to ascent

upto three-fourth of the TLC plate, it was taken out and air dried. The chromatogram was sprayed with 0.3 per cent ninhydrin, in acetone for the detection of free amino acids. It was then air dried at room temperature and later in a hot air oven. The Rf values of spots developed were calculated after marking their position with a pencil against the standard.

3.3 Immunodetection

3.3.1 Direct antigen coating-enzyme linked immunosorbent assay (DAC-ELISA)

ELISA for the detection of cowpea viruses was done using monoclonal antibodies received from International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria. Antigen was obtained from the virus culture maintained in the glass house through mechanical transmission from diseased to healthy plants. Diseased samples were taken from field also. The serodiagnosis of virus was carried out following the procedure described by Huguenot *et al.* (1992).

Direct antigen coating ELISA (DAC-ELISA)

The healthy and infected leaf samples were ground separately in phosphate buffered saline-tween (PBS-T) (1 : 5 w/v). The homogenate was centrifuged at 5000 rpm for 10 minutes at 4^0C. Samples were dispensed at the rate of 100 µl into Nunc immunological plates. The treatments were replicated twice. After incubation for 2 h at 37^0C the wells were washed with PBS-T three times each for a duration of three minutes. The plates were tapped on a blotting paper to remove PBS-T. Blocking was done with 100 µl of one per cent BSA for 30 minutes at 37^0C. After incubation blocking agent was removed, plates were washed with PBS-T as before. Monoclonal antibodies

10G5 and 16G5 which were specific to BlCMV and monoclonal antibodies 5H5, 7D9 and 6C10 specific to CABMV were used as detecting antibodies. The antibodies at 1 : 10000 dilution in PBS-T were added and incubated overnight at 4^{0}C. The plates were washed with PBS-T and then treated with 100 µl of alkaline phosphatase conjugated antimouse immunoglobulin diluted in PBS-T (10^{-4}) and incubated for 2 h at 37^{0}C. Wells were washed with PBS-T as before. The substrate p-nitrophenyl phosphate (p-NPP) in diethanolamine buffer (1 mg per ml) was added to each well (100 µl per well) and incubated for one hour at 37^{0}C. Reaction was stopped by adding 50 µl of four per cent NaOH. The absorbance was measured at 405 nm in an ELISA reader (ECIL, MS5608).

Buffer for ELISA

1. Phosphate buffered saline-tween (PBS-T) pH 7.4

NaCl	–	8.0 g
KH_2PO_4	–	0.2 g
Na_2HPO_4	–	1.1 g
KCl	–	0.2 g
NaN_3	–	0.2 g

In one litre water

Tween-20 (0.05 per cent) – 0.5 ml

2. Substrate buffer - Diethanol amine buffer (pH 9.8)

Diethanolamine	–	97 ml
H_2O	–	800 ml
NaN_3	–	0.2 g

Add 1N HCl to give pH 9.8

Results

4. RESULTS

4.1 Screening the sources of virus resistance

Sixty six varieties were screened for their reaction to BlCMV infection under glasshouse condition. Assessment of the degree of resistance in cowpea cultivar was done based on 0 – 5 scale proposed by Rajamony *et al.* (1990). The vulnerability index (V.I.) was calculated and all 66 varieties were grouped into five grades (Plate 1).

Grade	Vulnerability index (V.I.)	Reaction category	Number of genotypes
I	0	Resistant (R)	4
II	1 – 25	Medium resistant (MR)	29
III	26 – 50	Medium susceptible (MS)	27
IV	51 – 75	Susceptible (S)	6
V	76 and above	Highly susceptible (HS)	Nil

The vulnerability index varied from 0-65.00. The lowest index was observed for four accessions viz., Co-6, Culture-9, accession 124 and accession 180 which were grouped as resistant. Maximum vulnerability index recorded was 65.00 for accession 173. Among the accessions screened, four were resistant to BlCMV, twenty nine moderately resistant, twenty seven moderately susceptible and six susceptible. None of the accessions were found to be highly susceptible (Table 1).

Table 1 Reaction of cowpea germplasm to BlCMV under glass house condition

Sl. No.	Collections	No. of plants	Vulnerability index	Range of score	Grade
1	Culture 9	9	0	-	R
2	Pusa Phalguni	6	25	0-3	MR
3	CoVU-623	11	40	0-4	MS
4	GC-8929	9	58.3	1-4	S
5	GC-3	9	25	0-2	MR
6	GAZC	9	8.5	0-1	MR
7	Krishnamani	11	9.25	0-1	MR
8	V-130	8	6.25	0-1	MR
9	C-152	7	20.0	1-2	MR
10	CAZ-9821	7	10.7	0-1	MR
11	Kanakamony	13	13.8	1-3	MR
12	Pallichal local	9	35.0	1-2	MS
13	CO-6	10	0	-	R
14	Sharika	9	44.4	4-2	MS
15	Malika	8	31.2	0-2	MS
16	CO-4	7	10.7	0-2	MR
17	161	5	5	0-1	MR
18	115	5	30	1-2	MS
19	168	4	50	0-2	MS
20	95	4	37.5	0-2	S
21	180	4	0	-	R
22	92	6	10	0-1	MR
23	29	5	50	0-2	MS
24	25	5	45	0-2	MS
25	89	5	31.25	0-2	MS
26	A-10	5	16.66	0-1	MR
27	48	5	15.0	0-2	MR
28	MP-1	5	40	1-3	MS
29	MP-2	6	29.16	1-2	MS
30	56	8	18.75	0-1	MR
31	12	4	8.3	0-1	MR
32	13	5	25	0-2	MR
33	73	4	43.75	1-3	MS
34	178	4	25	0-2	MR
35	84	6	20	0-2	MR
36	18	4	31.25	0-2	MS
37	186	4	37.5	1-2	MS
38	19	4	12.5	0-1	MR
39	156	4	41.6	0-3	MS
40	90	3	50	0-3	MS
41	124	4	0	-	R
42	134	7	25	0-2	MR

Table 1 Contd...

Sl. No.	Collections	No. of plants	Vulnerability index	Range of score	Grade
43	14	6	58	0-3	S
44	148	5	18.75	0-2	MR
45	149	3	41.6	1-2	MS
46	99	6	40.0	0-2	MS
47	173	5	65	2-3	S
48	69	8	46.8	1-3	MS
49	202774	8	53.1	1-3	S
50	202778	9	38.8	1-3	MS
51	202710	7	9.4	0-1	MR
52	202815	10	22.5	0-1	MR
53	201081	8	15.6	0-1	MR
54	202818	8	9.3	0-1	MR
55	202780	10	32.5	1-3	MS
56	202781	11	61.1	1-3	S
57	202797	9	50	1-3	MS
58	202781	11	61.1	1-3	MS
59	202779	9	50	0-3	MS
60	202829	9	3.12	0-1	MR
61	202850	7	14.2	0-2	MR
62	202918	4	6.2	0-1	MR
63	202880	5	40	1-3	MS
64	202833	7	10	0-1	MR
65	202852	9	37.5	1-2	MS
66	202861	6	50	1-3	MS

Plate 1 Disease scoring

0 : no symptom

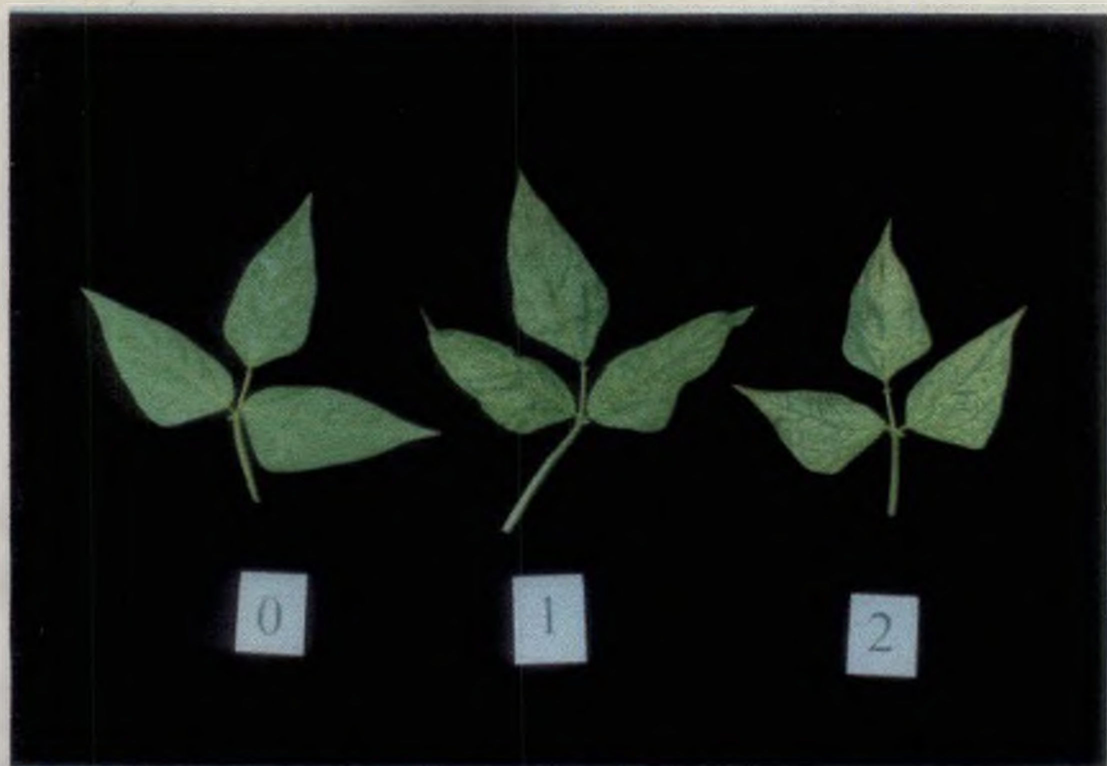
1 : Vein clearing

2 : Light and dark green mottling

3 : Blistering

4 : Distortion

5 : Stunting of plant


0
1
2

3
4
5

Cross combinations of Co-6, Sharika, Malika and Pallichal local were used for hybridization to develop F_1 hybrids (Plates 2 – 9). Following six cross combinations were tried.

1. Sharika x Co-6
2. Co-6 x Sharika
3. Malika x Co-6
4. Co-6 x Malika
5. Pallichal local x Co-6
6. Co-6 x Pallichal local

The F_1 hybrid seeds were collected for further evaluation.

4.2 Biochemical changes of host-pathogen interaction

4.2.1 Estimation of total carbohydrate

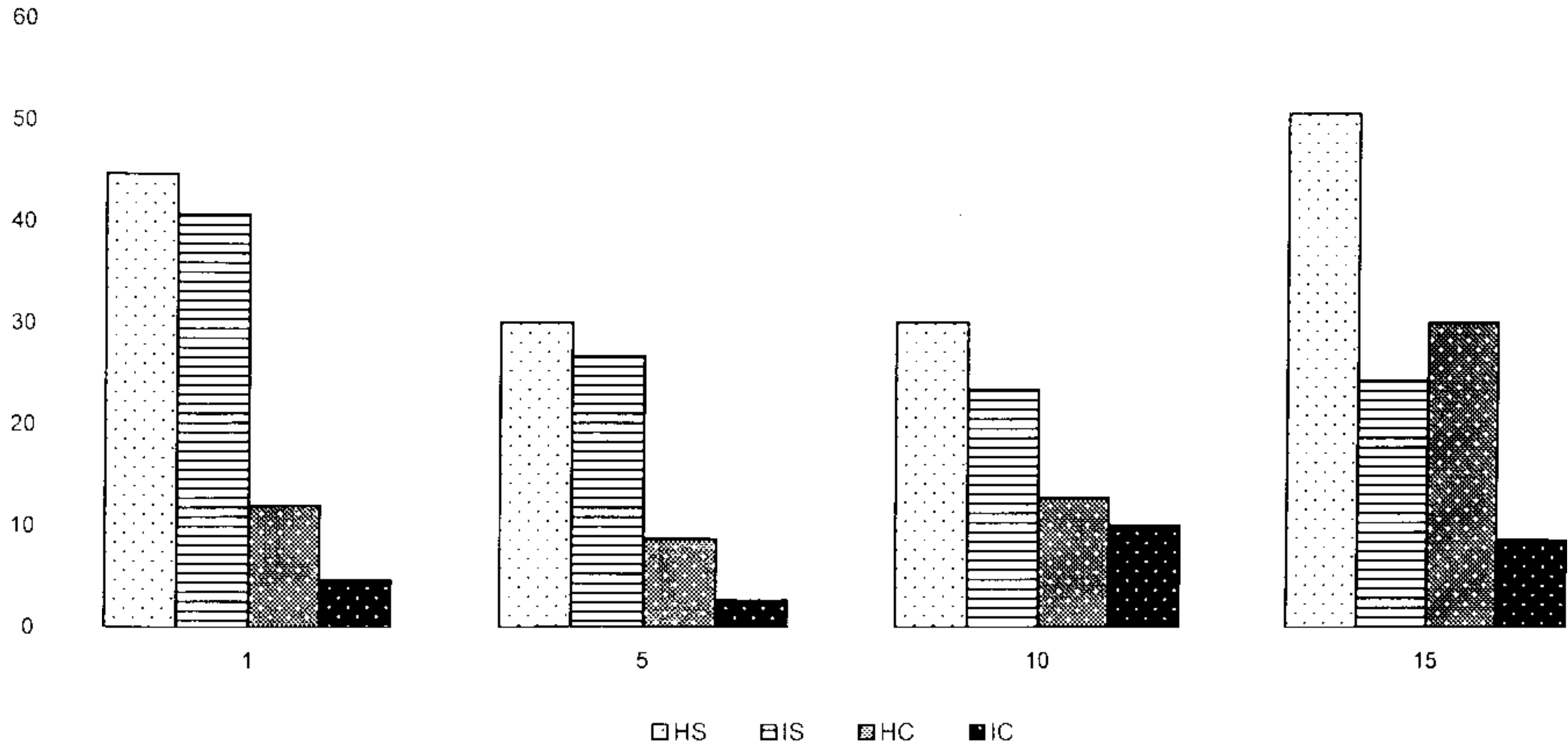
The estimation was done according to the procedure given by Hedge and Hofreiter (1954). The result indicated that there was significant difference between carbohydrate level in susceptible and resistant variety and there was lower levels of total carbohydrate in resistant plants (Table 2, Fig. 1). In healthy susceptible plants a value of 44.67 mg g^{-1} was recorded at one day after inoculation (DAI). Thereafter it reduced to 30 mg g^{-1} at 5 DAI and remained stable at 10 DAI and it showed an increase to 50.67 mg g^{-1} at 15 DAI. Following inoculation total carbohydrate content of susceptible variety reduced to 23.33 mg g^{-1} at 10 DAI from 40.67 mg g^{-1} at one DAI and then it increased to 24.33 mg g^{-1} at 15 days after inoculation.

Table 2 Changes in total carbohydrate content of cowpea leaves in response to BlCMV inoculation

Days after inoculation	Change in carbohydrate content (mg g^{-1} fresh weight of tissue)			
	Susceptible variety, Sharika		Resistant variety, Co-6	
	Healthy	Inoculated	Healthy	Inoculated
1	44.67	40.67	12.00	4.67
5	30.00	26.67	8.67	2.67
10	30.00	23.33	12.67	10.00
15	50.67	24.33	30.00	8.67

CD values – 3.42
CD variety x treatment interaction - 4.83
CD variety x treatment x period interaction – 9.66

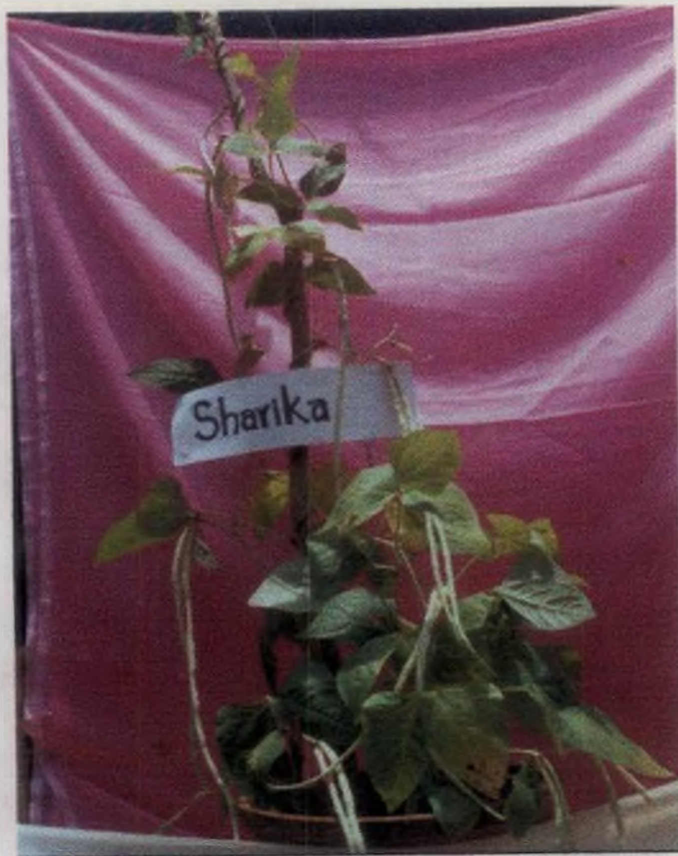
Fig. 1 Changes in total carbohydrate content of cowpea leaves in response to BICMV inoculation



HS - Healthy Sharika (Susceptible variety)
IS - Inoculated Sharika (Susceptible variety)
HC - Healthy Co-6 (Resistant variety)
IC - Inoculated Co-6 (Resistant variety)

Plate 2 Sharika – variety used in hybridization

Plate 3 Pod character of Sharika


Sharika

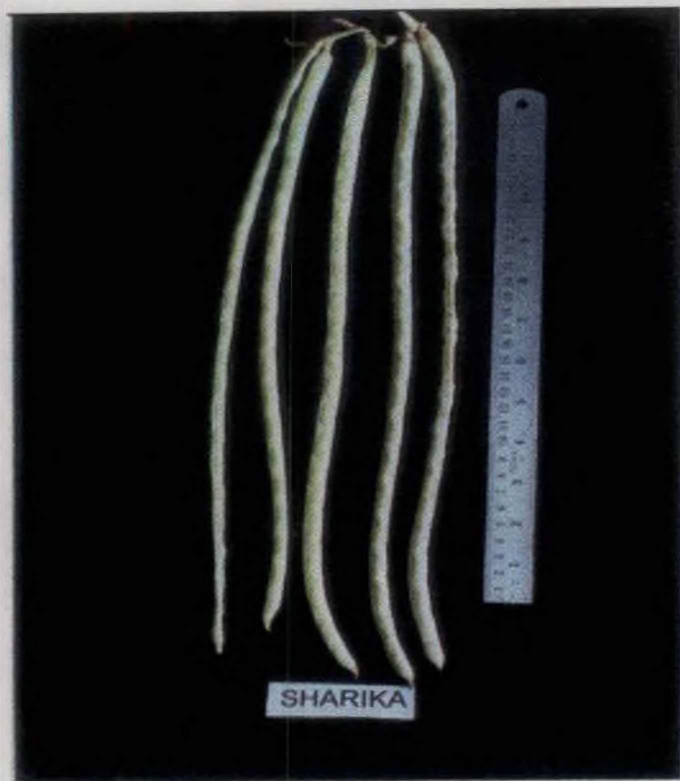

SHARIKA

Plate 4 Malika – variety used in hybridization

Plate 5 Pod character of Malika

Malika

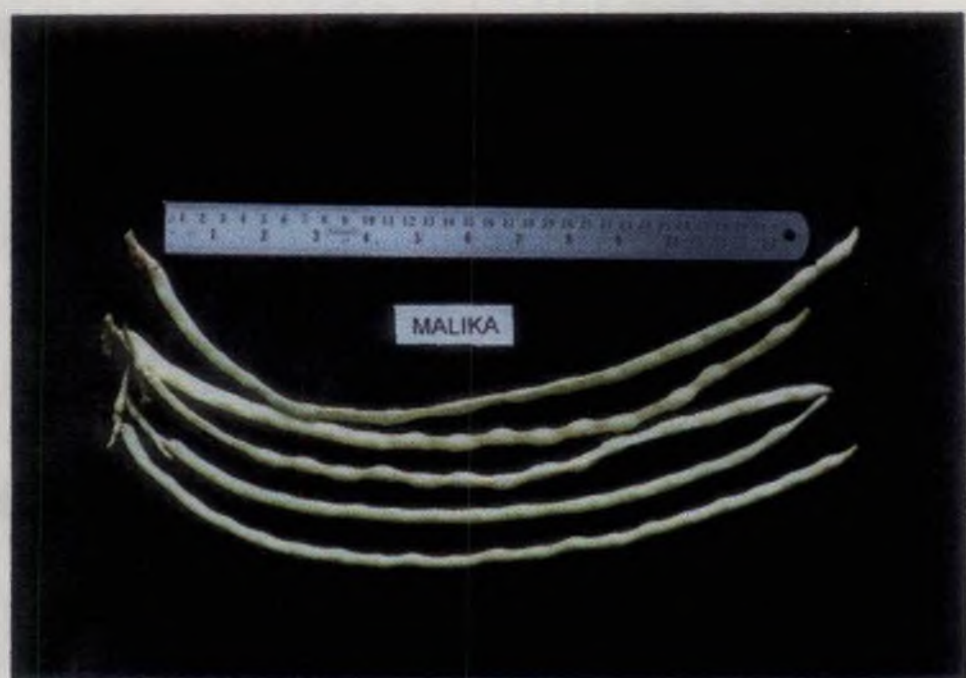

MALIKA

Plate 6 Pallichal local – variety used in hybridization

Plate 7 Pod character of Pallichal local

Pallichal local

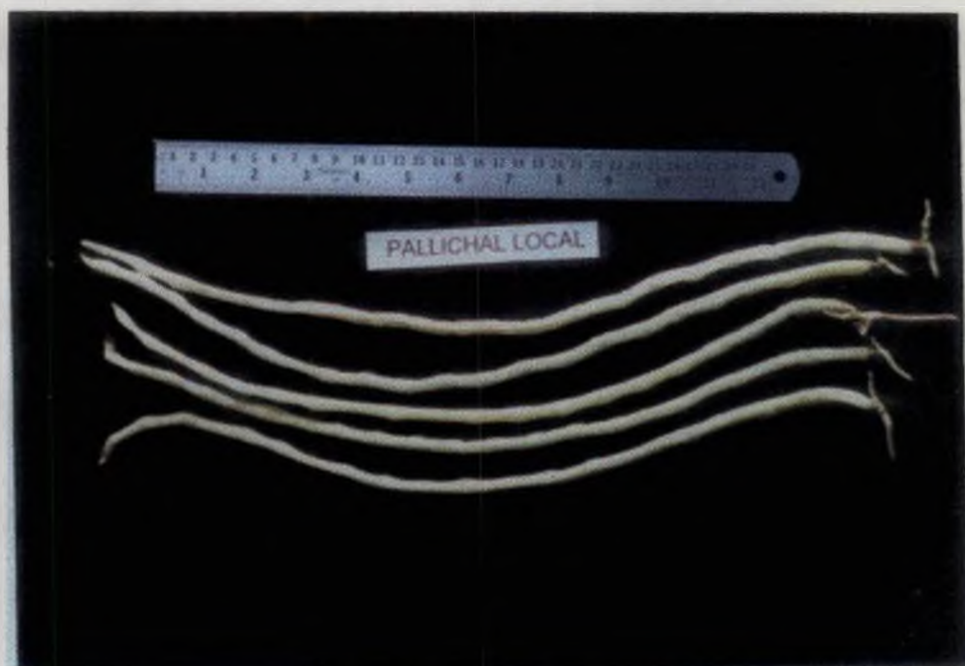

PALLICHAL LOCAL

Plate 8 Co-6 – Resistant source for hybridization obtained from TNAU

Plate 9 Pod character of Co-6

CO-6

CO – 6

In the resistant variety Co-6 the total carbohydrate content was lower when compared to susceptible variety. In the case of uninoculated control of resistant variety, the carbohydrate content recorded 12 mg g^{-1} at one DAI progressively increased to 30.00 mg g^{-1} at 15 DAI.

Upon inoculation, resistant variety also showed a reduction in level of total carbohydrate content. Table 2 indicate that the carbohydrate content of inoculated resistant genotype recorded 4.67 mg g^{-1} at one DAI and it increased to 10.00 mg g^{-1} at 10 DAI and then reduced to 8.67 mg g^{-1} at 15 DAI.

4.2.2 Estimation of chlorophyll

The total chlorophyll content was estimated as per the procedure described by Arnon (1949). The results for monitoring changes in chlorophyll content of susceptible and resistant genotypes indicated that it was significantly greater in uninoculated control when compared to inoculated genotypes. On inoculation of susceptible genotype with BlCMV a significant reduction in chlorophyll content was noticed, and it was minimum (0.62 mg g^{-1}) at 10 DAI (Table 3).

It was observed that in resistant variety, uninoculated control and in inoculated plants, the level of chlorophyll was on par (Fig. 2). There was a progressive increase from 1.13 mg g^{-1} at one DAI to 1.44 mg g^{-1} at 15 DAI except for 5 DAI which showed a reduction of 0.85 mg g^{-1}. Upon inoculation also no significant change in healthy and inoculated plants was observed. A maximum value of 1.41 mg g^{-1} at 15 DAI and minimum value of 0.83 mg g^{-1} at one DAI and was on par with five DAI.

Table 3 Changes in chlorophyll content of cowpea leaves in response to BlCMV inoculation

Days after inoculation	Changes in chlorophyll content (mg g^{-1} fresh weight)											
	Susceptible variety, Sharika						Resistant variety, Co-6					
	Healthy			Inoculated			Healthy			Inoculated		
	a	b	Total	a	b	Total	a	b	Total	a	b	Total
1	1.23	0.33	1.56	0.63	0.17	0.79	0.99	0.43	1.13	0.74	0.11	0.85
5	0.66	0.17	0.82	0.58	0.14	0.77	0.73	0.28	0.85	0.78	0.06	0.83
10	0.82	0.36	1.18	0.50	0.11	0.62	0.90	0.46	1.36	1.06	0.30	1.36
15	0.73	0.36	0.99	0.57	0.16	1.06	1.14	0.30	1.44	1.08	0.33	1.41

Chlorophyll a

CD values – 0.04
CD variety x treatment interaction – 0.06
CD variety x treatment x period interaction – 0.12

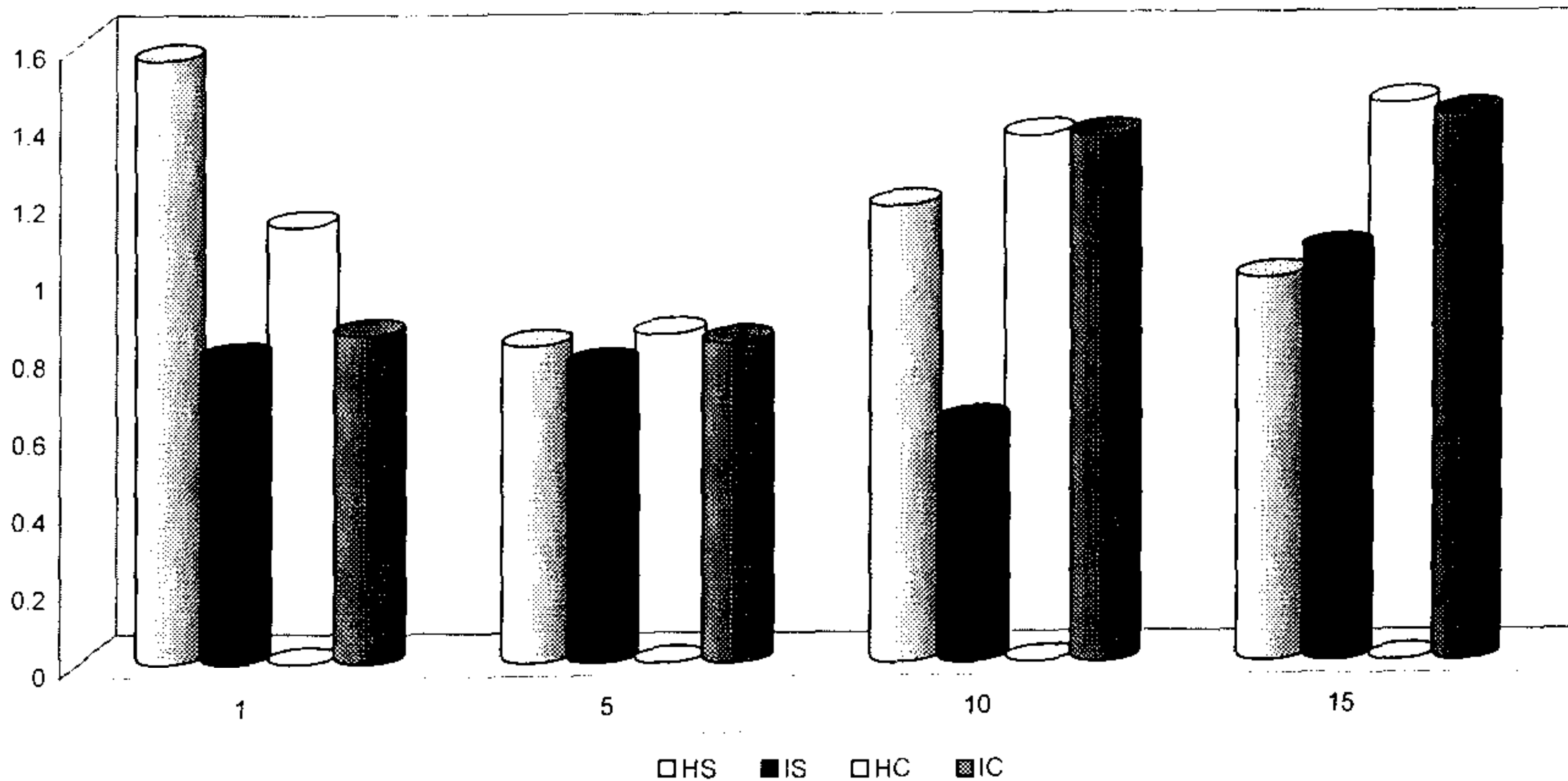
Chlorophyll b

CD values – 0.09
CD variety x treatment interaction – 0.13
CD variety x treatment x period interaction – 0.25

Total chlorophyll

CD values – 0.10
CD variety x treatment interaction – 0.14
CD variety x treatment x period interaction – 0.28

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



HS - Healthy Sharika (Susceptible variety)
IS - Inoculated Sharika (Susceptible variety)

HC - Healthy Co-6 (Resistant variety)
IC - Inoculated Co-6 (Resistant variety)

4.2.3 Estimation of phenol

The estimation was done according to the procedure given by Bray and Thorpe (1954). There was progressive increase in total phenol content of healthy plants with increase in plant age in susceptible variety (Sharika) (Fig. 3). However there was significantly lower levels of total phenol in resistant variety Co-6 than in susceptible variety. Due to infection with BlCMV the total phenolics of susceptible plants was increased substantially upto 5 DAI. The increase noticed was 129.97 $\mu g\ g^{-1}$ which was maximum at five DAI (Table 4). Subsequently the total phenol content was found to decrease registering 94.4 $\mu g\ g^{-1}$ at 10 DAI, there after there was a gradual increase. The phenol content of resistant variety on inoculation remained constant.

4.2.4 Estimation of protein

Estimation of protein was carried out as per the procedure given by Bradford (1976). The results (Table 5, Fig. 4) indicated that, inoculation of susceptible plants with BlCMV caused a significant difference in total soluble protein content. The total soluble protein content was found higher in case of inoculated susceptible plants. The level of protein content increased with age of plant (Fig. 4). The protein content increased from 106 $\mu g\ g^{-1}$ at one DAI and reached a maximum of 140.67 $\mu g\ g^{-1}$ at 15 DAI. The uninoculated plants also showed the same trend. At one DAI it was 52 $\mu g\ g^{-1}$ which increased to 91.33 $\mu g\ g^{-1}$.

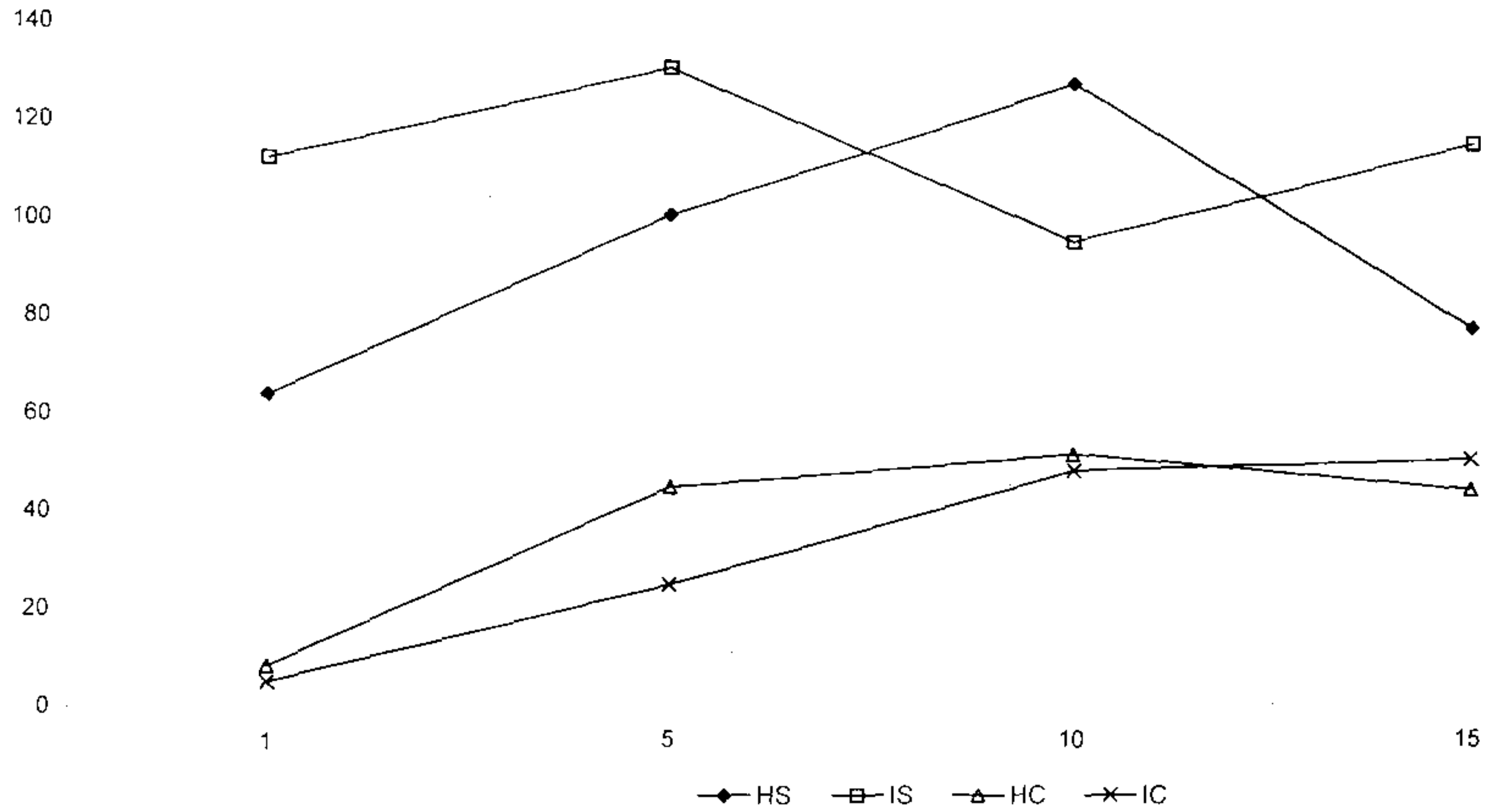
In resistant plant upon inoculation, also showed an increase in total soluble proteins. It was minimum (108 $\mu g\ g^{-1}$) and maximum (179.33 $\mu g\ g^{-1}$)

Table 4 Changes in total phenol content of cowpea leaves in response to BlCMV inoculation

Days after inoculation	Changes in total phenol content (μg g^{-1} fresh weight of tissue)			
	Susceptible (Sharika)		Resistant (Co-6)	
	Healthy	Inoculated	Healthy	Inoculated
1	63.87	112.20	8.30	4.97
5	99.97	129.97	44.43	24.40
10	126.63	94.40	51.09	47.74
15	76.63	114.40	43.80	50.00

CD values – 4.37
CD variety x treatment interaction – 6.18
CD variety x treatment x period interaction – 12.36

Fig. 3 Changes in total phenol content of cowpea leaves in response to BICMV inoculation



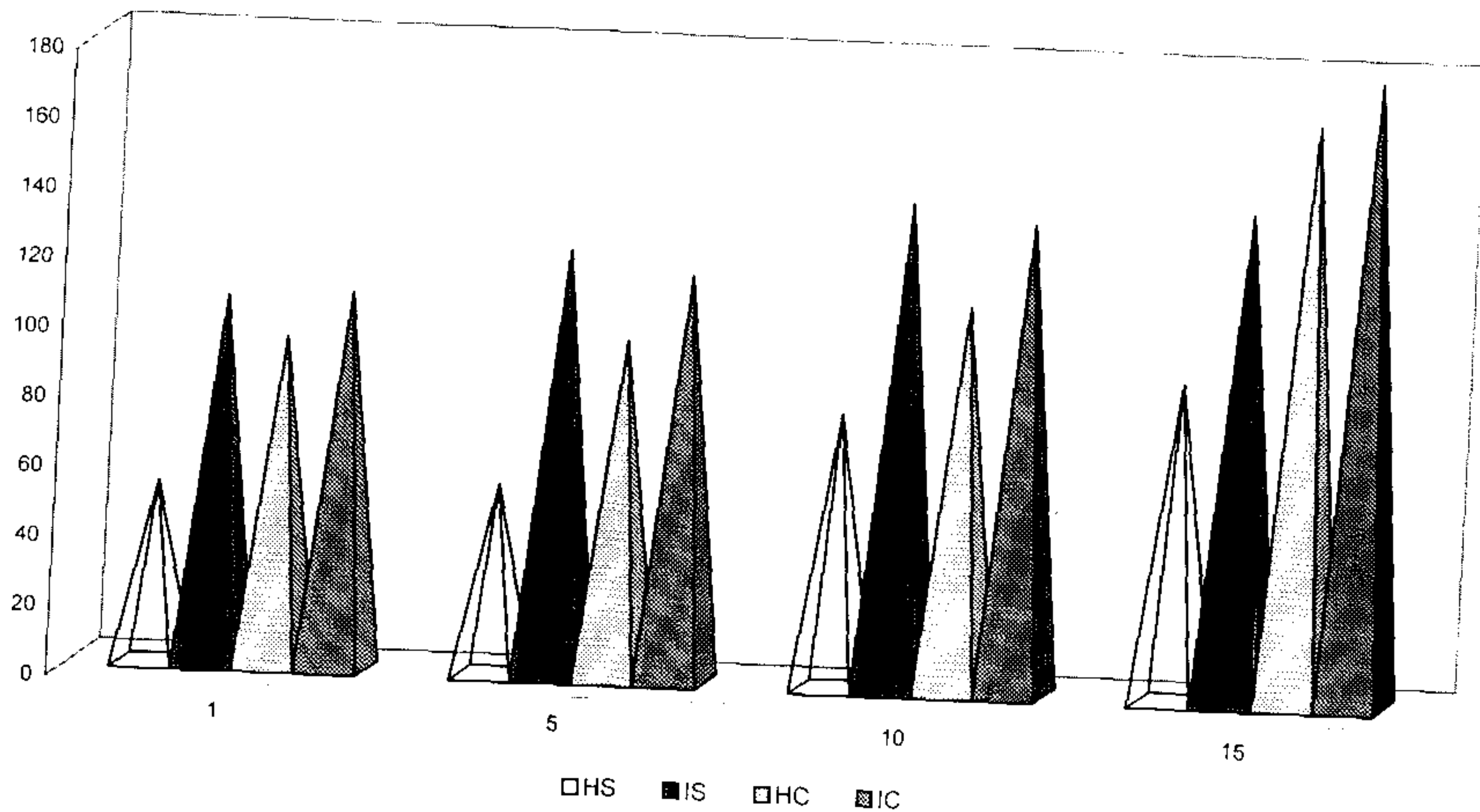
HS - Healthy Sharika (Susceptible variety)
IS - Inoculated Sharika (Susceptible variety)
HC - Healthy Co-6 (Resistant variety)
IC - Inoculated Co-6 (Resistant variety)

Table 5 Changes in total soluble protein content of cowpea leaves in response to BlCMV inoculation

Days after inoculation	Changes in soluble protein content (μg^{-1} fresh weight of tissue)			
	Susceptible variety, Sharika		Resistant variety, Co-6	
	Healthy	Inoculated	Healthy	Inoculated
1	52.00	106.00	94.67	108.00
5	54.67	122.67	97.33	116.67
10	78.67	140.00	110.67	135.33
15	91.33	140.67	166.67	179.33

CD values – 9.64
CD variety x treatment interaction – 13.63
CD variety x treatment x period interaction – 27.25

Fig. 4 Changes in total protein content of cowpea leaves in response to BICMV inoculation



HS - Healthy Sharika (Susceptible variety)
IS - Inoculated Sharika (Susceptible variety)

HC - Healthy Co-6 (Resistant variety)
IC - Inoculated Co-6 (Resistant variety)

at one DAI and 15 DAI respectively. The corresponding values in uninoculated control was 94.67 and 166.67 $\mu g\ g^{-1}$.

4.2.5 Defence related enzymes

4.2.5.1 Peroxidase

Estimation of peroxidase activity was carried out as per the procedure given by Srivastava (1987). There was a progressive increase in peroxidase activity with age of the plant, in both healthy and inoculated resistant and susceptible variety. The peroxidase activity was found higher in case of inoculated plants than in healthy susceptible variety, Sharika. Peroxidase activity increased progressively from 0.53 $min^{-1}\ g^{-1}$ at one day to 2.67 $min^{-1}\ g^{-1}$ at 15 day in case of healthy plants and 0.60 $min^{-1}\ g^{-1}$ at one day to 3.07 $min^{-1}\ g^{-1}$ at 15 day in case of inoculated plants (Table 6).

In resistant variety the peroxidase activity was found higher than that of susceptible variety (Fig. 5). In case of healthy resistant plants the activity was found progressively increasing form 0.93 $min^{-1}\ g^{-1}$ at one day to 4.52 $min^{-1}\ g^{-1}$ at 15 DAI, whereas in inoculated resistant plants the peroxidase activity was observed to be higher and it increased form 1.42 at one DAI to 5.47 $min^{-1}\ g^{-1}$ at 15 DAI.

4.2.5.2 Polyphenol oxidase activity

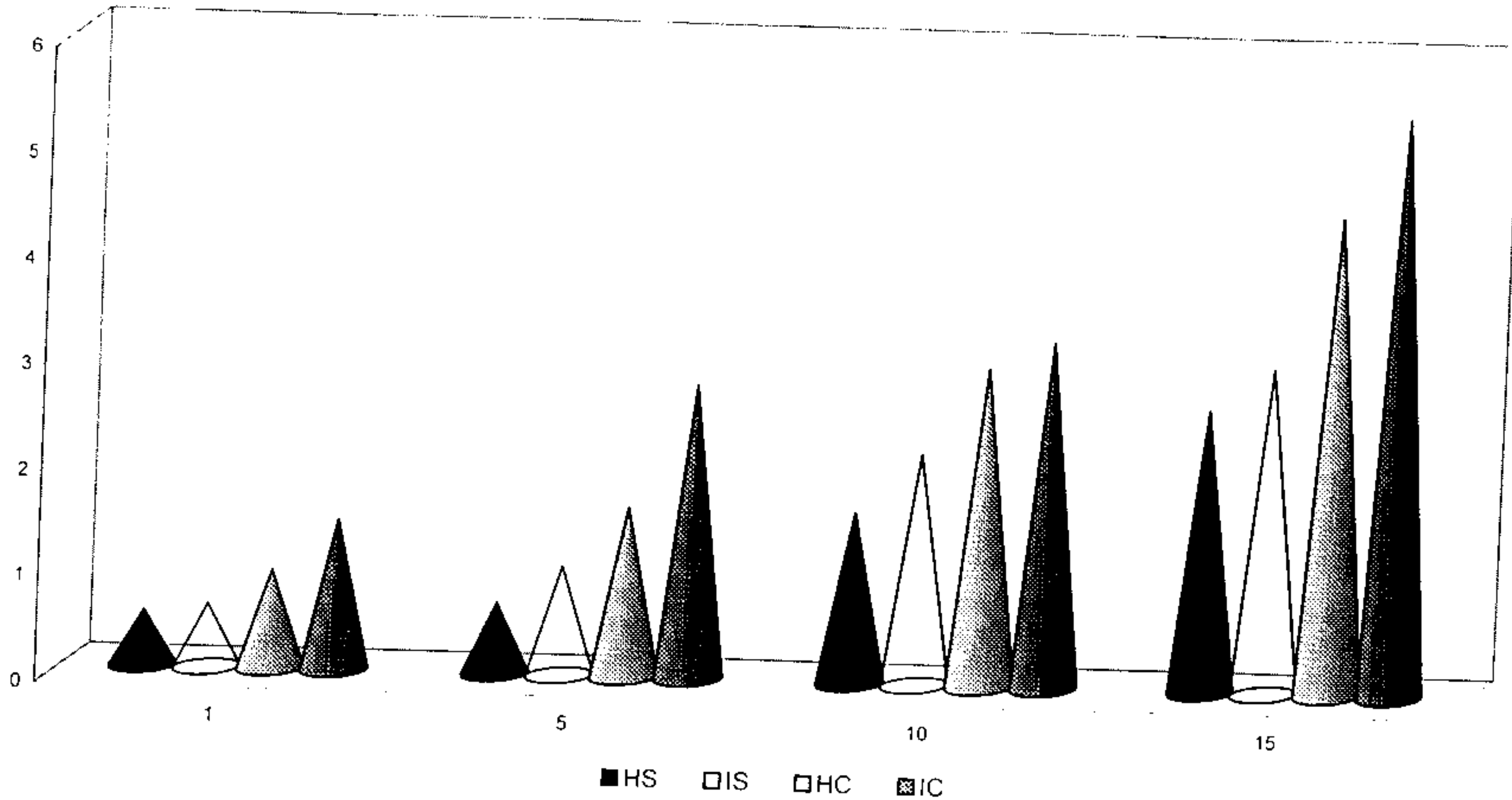
Estimation of polyphenol oxidase activity was conducted as per the procedure suggested by Mayer *et al.* (1965). The results in Table 7, Fig. 6 indicated that there was significant difference between susceptible and resistant variety and the activity was higher in the case of resistant variety. In healthy susceptible plant the activity was found to be lower when compared to

Table 6 Changes in peroxidase activity in cowpea plants in response to BlCMV inoculation

Days after inoculation	Peroxidase activity (change in absorbance (min^{-1} g^{-1} fresh weight)			
	Susceptible variety, Sharika		Resistant variety, Co-6	
	Healthy	Inoculated	Healthy	Inoculated
1	0.53	0.60	0.93	1.42
5	0.67	1.03	1.60	2.78
10	1.62	2.19	3.01	3.27
15	2.67	3.07	4.52	5.47

CD values – 0.34
CD variety x treatment interaction – 0.48
CD variety x treatment x period interaction – 0.96

Fig. 5 Changes in peroxidase activity in cowpea plants in response to BICMV inoculation



HS - Healthy Sharika (Susceptible variety)
IS - Inoculated Sharika (Susceptible variety)

HC - Healthy Co-6 (Resistant variety)
IC - Inoculated Co-6 (Resistant variety)

Table 7 Changes in polyphenol oxidase activity in cowpea plants in response to BlCMV inoculation

Days after inoculation	Activity of polyphenol oxidase (changes in absorbance min^{-1} g^{-1} fresh weight)			
	Susceptible (Sharika)		Resistant (Co-6)	
	Healthy	Inoculated	Healthy	Inoculated
1	0.03	0.05	0.12	0.12
5	0.02	0.09	0.11	0.13
10	0.01	0.11	0.12	0.16
15	0.02	0.14	0.09	0.05

CD values – 0.34
CD variety x treatment interaction – 0.48
CD variety x treatment x period interaction – 0.96

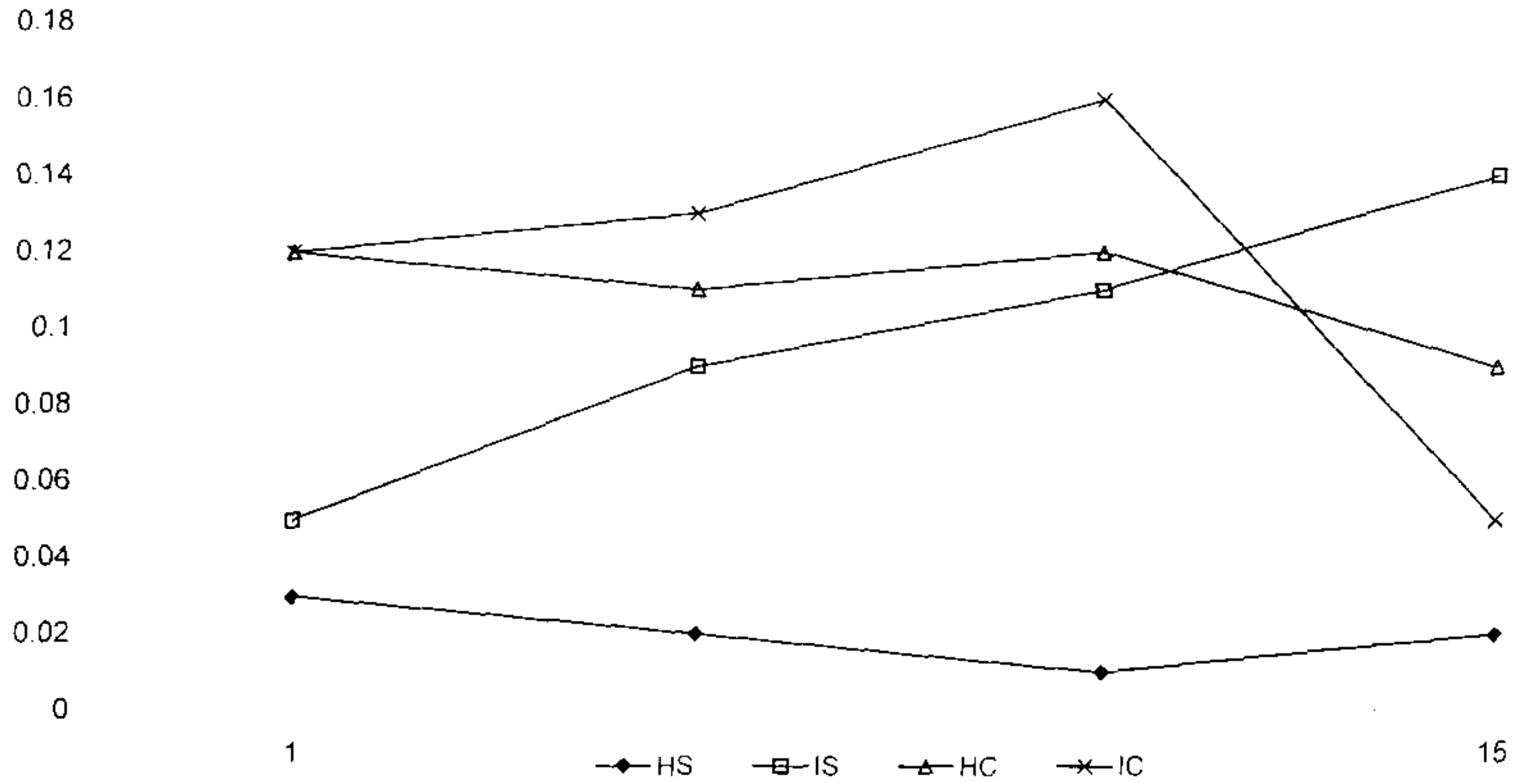


Fig. 6 Changes in polyphenol oxidase activity in cowpea plants in response to BlCMV inoculation

HS - Healthy Sharika (Susceptible variety)
IS - Inoculated Sharika (Susceptible variety)
HC - Healthy Co-6 (Resistant variety)
IC - Inoculated Co-6 (Resistant variety)

inoculated plants. Upon inoculation the activity progressively increased from 0.05 min^{-1} g^{-1} at one DAI to 0.14 min^{-1} g^{-1} at 15 DAI but in uninoculated control the activity gradually decreased to 0.01 min^{-1} g^{-1}at 10 DAI from 0.03 min^{-1} g^{-1} and thereafter it increased to 0.02 min^{-1} g^{-1}.

In resistant variety, Co-6 no significant difference was observed between healthy and inoculated plants. The enzyme activity was stable with increase in plant age in case of uninoculated resistant plants. Following inoculation, the enzyme activity increased form 0.12 min^{-1} g^{-1} at one day to 0.16 min^{-1} g^{-1} at 10 DAI and thereafter it decreased to 0.05 min^{-1} g^{-1}.

4.2.5.3 Phenylalanine ammonialyase

PAL activity was estimated as per the procedure developed by Dickerson *et al.* (1984). Results indicated that PAL activity was enhanced in case of inoculated susceptible plants when compared to healthy (Table 8). The enzyme activity of 1485 nm g^{-1} min^{-1} was observed 5 DAI in healthy susceptible plant then it gradually dropped to 825.58 nm g^{-1} min^{-1} at 15 DAI. The activity was higher in case of inoculated susceptible plants and recorded a maximum value of 1827.62 nm g^{-1} min^{-1} at 5 DAI and then there was a sudden drop to 654.20 nm g^{-1} min^{-1} at 15 DAI.

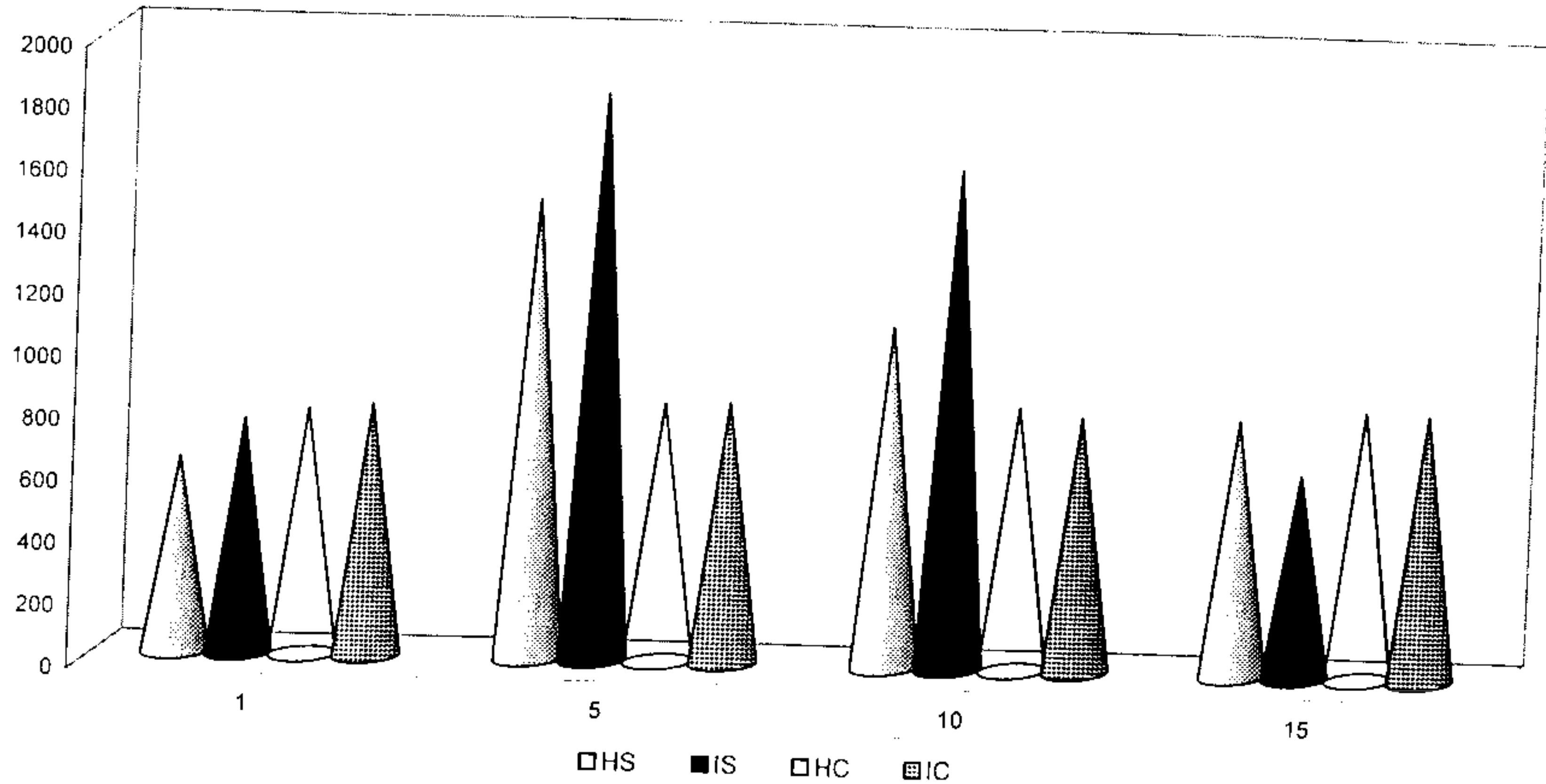
Results revealed that in resistant variety Co-6, there was no significant difference between healthy and inoculated plants. The enzyme activity was comparatively lower when compared to susceptible variety (Fig. 7). Inoculation of resistant plant could not alter any change in the PAL activity. The activity of the enzyme in both healthy and inoculated plants remained constant at all periods of sampling.

Table 8 Changes in phenylalanine ammonialyase (PAL) activity in cowpea plants in response to BlCMV inoculation

Days after inoculation	Activity of PAL ($\mu g\ g^{-1}\ min^{-1}$)			
	Susceptible variety Sharika		Resistant variety Co-6	
	Healthy	Inoculated	Healthy	Inoculated
1	630.87	758.05	796.12	813.43
5	1485.00	1827.62	834.210	841.12
10	1099.04	1609.55	848.04	820.35
15	825.58	654.20	861.88	854.96

CD values – 114.79
CD variety x treatment interaction – 162.34
CD variety x treatment x period interaction – 229.58

Fig. 7 Changes in phenylalanine ammonialyase activity in cowpea plants in response to BICMV inoculation



HS - Healthy Sharika (Susceptible variety)
IS - Inoculated Sharika (Susceptible variety)

HC - Healthy Co-6 (Resistant variety)
IC - Inoculated Co-6 (Resistant variety)

4.2.6 Eletrophoretic separation of soluble proteins

Polyacrylamide gel electrophoresis was done for proteins following the protocol coined by Laemmeli (1970). R_f value of the band produced were calculated. SDS-PAGE of protein profiles of healthy cowpea plants and plants following inoculation with BlCMV (Plate 10a) produced five proteins with R_f values 0.56, 0.61, 0.65, 0.87 and 0.95 in both healthy and diseased plants. Identical protein profile was observed in both healthy and disease samples. The intensity of the bands were more prominent in inoculated plants comparable to uninoculated plants (Table 9.1, Fig. 8).

In another experiment (Plate 10b) samples extracted from plants at 15 DAI and from healthy plants of same age showed that there was significant difference in the protein profile of inoculated and uninoculated control. Seven proteins with R_f values 0.32, 0.49, 0.57, 0.66, 0.69, 0.82 and 0.89 were present in inoculated plants when compared to five proteins in samples of uninoculated control (Table 9.2). The two novel proteins with R_f value 0.32 and 0.49 may be induced due to virus infection (Fig. 8).

4.2.7 Eletrophoretic analysis of isozymes

Native polyacrylamide gel electrophoresis was carried out for isozyme analysis of polyphenol oxidase (PPO). This experiment was performed to find the variability in cowpea varieties used for hybridization. The R_f values of each band was calculated and relative mobility were diagrammatically represented in Fig. 9. There was significant difference between the genotypes analysed. There were five isoforms of PPO with R_f values, 0.07, 0.10, 0.2, 0.25 and 0.55 for the susceptible variety Malika. Another susceptible variety

Table 9 Electrophoretic separation of soluble proteins

Table 9.1

R_f value	
Diseased	Healthy
0.56	0.56
0.61	0.61
0.65	0.65
0.87	0.87
0.95	0.95

Table 9.2

R_f value	
Diseased	Healthy
0.32	-
0.49	-
0.57	0.57
0.66	0.66
0.69	0.69
0.82	0.82
0.89	0.89

Table 10 Electrophoretic analysis of isozymes

R_f values			
Sharika	Co-6	Pallichal local	Malika
0.07	0.07	0.07	0.07
0.10	0.11	0.10	0.10
0.18	0.20	0.20	0.20
0.23	0.25	0.25	0.25
0.47	-	-	0.55

Fig.8. Protein profile studies of healthy and diseased cowpea plants

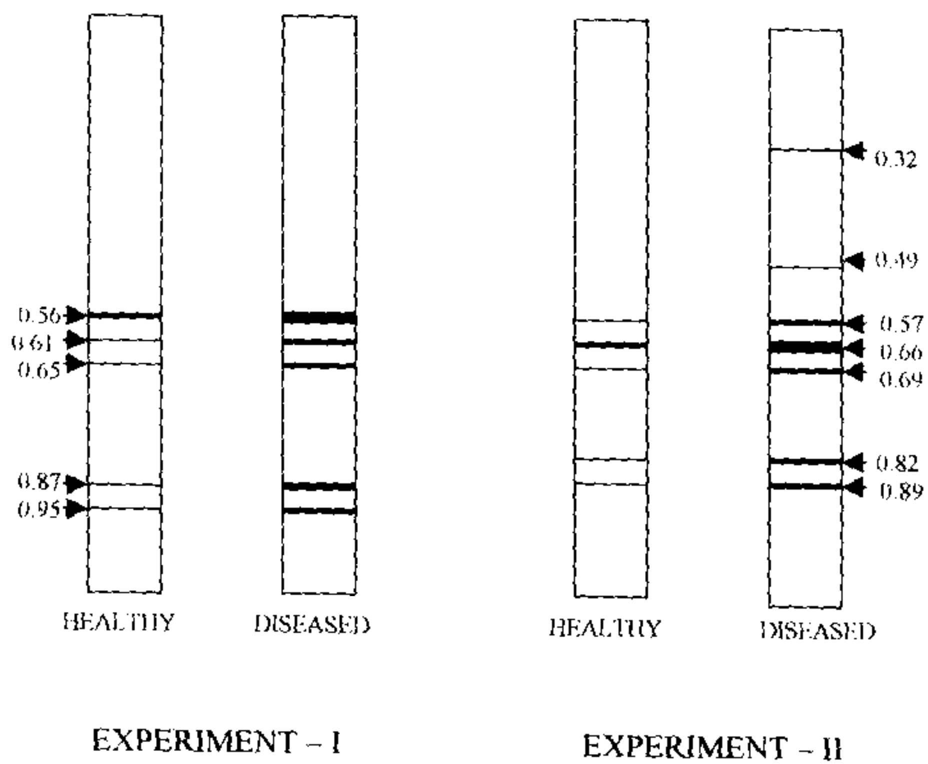
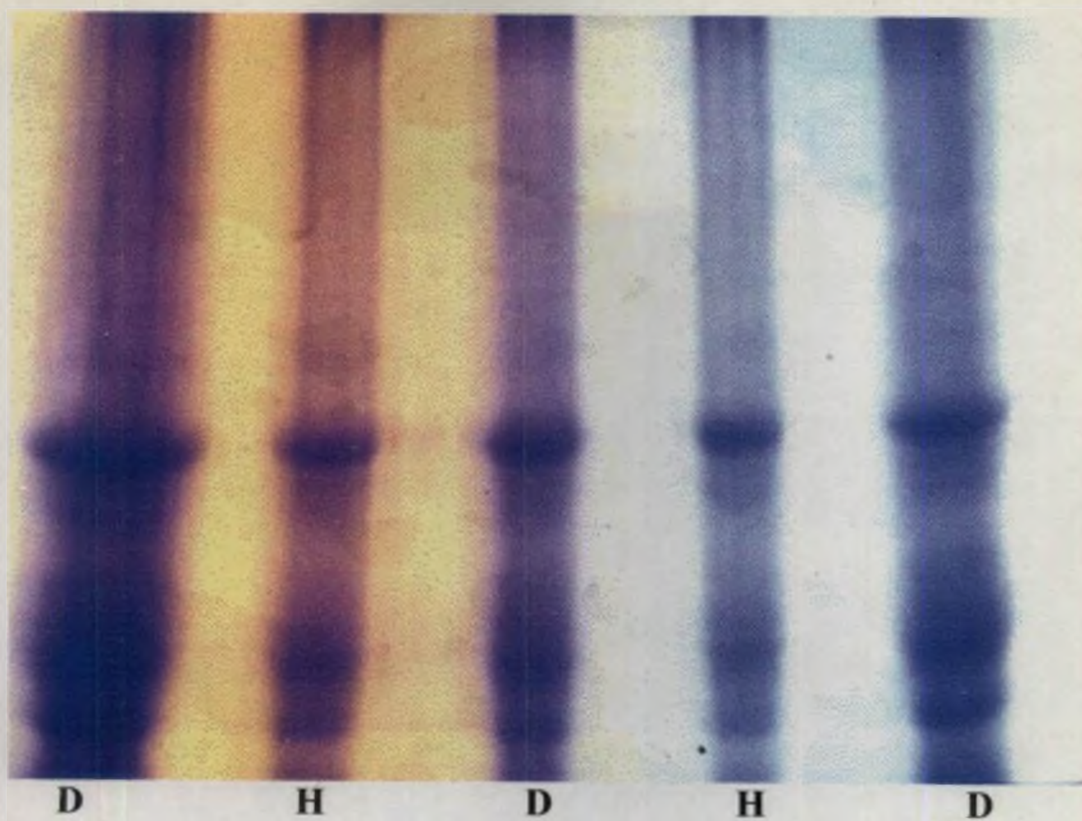


Plate 10 Protein profile of diseased and healthy samples in 12.5 per cent SDS-PAGE

Plate 10a. Experiment I

Plate 10b. Experiment II


D
H
D
H
D

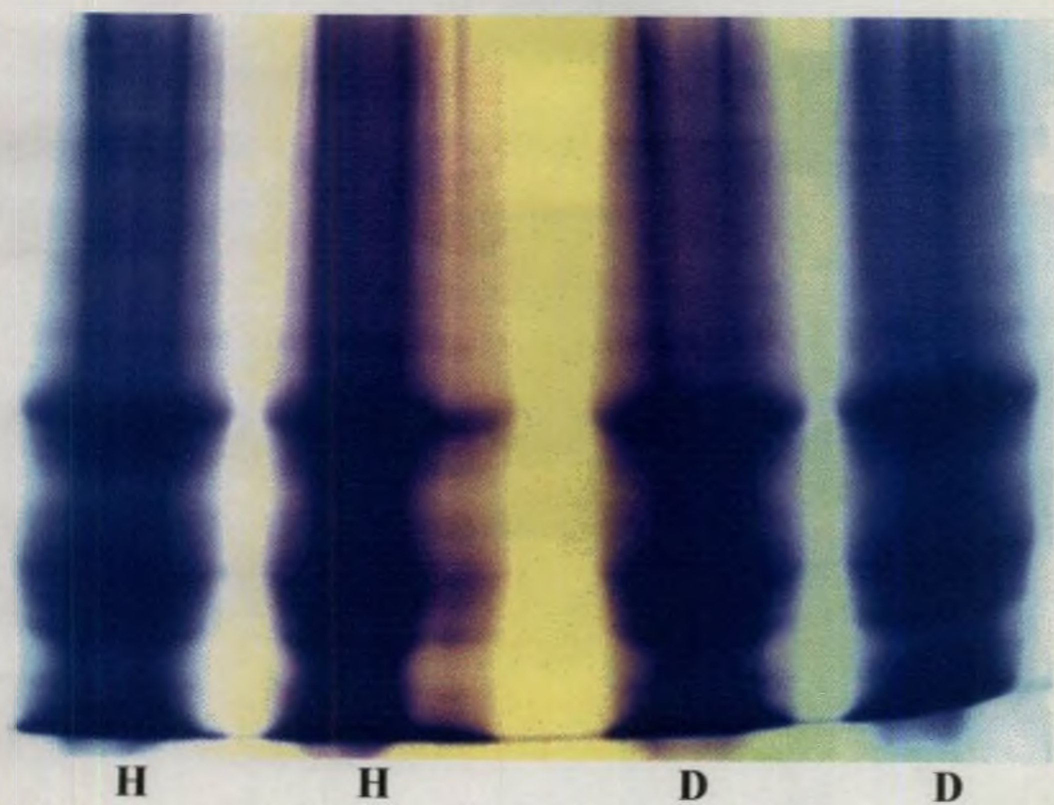
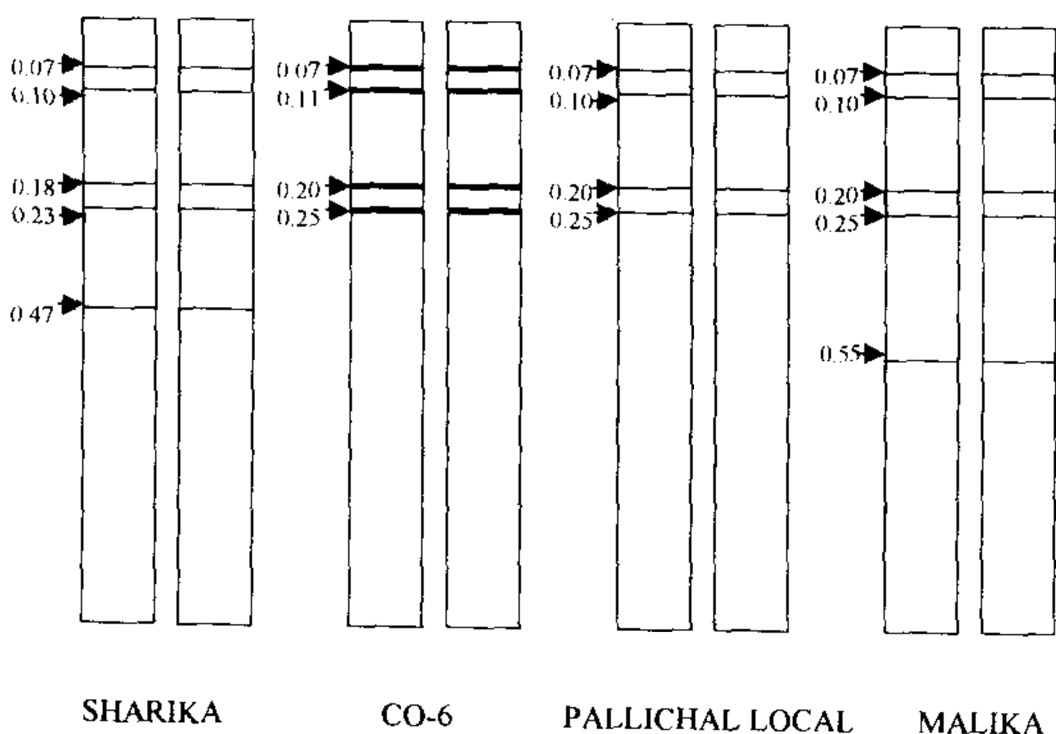

H
H
D
D

Fig. 9. Zymogram of Polyphenol oxidase of cowpea genotypes



171180C

Sharika also expressed five isoforms of PPO with R_f values 0.07, 0.10, 0.18, 0.23 and 0.47. The two genotypes Co-6 and Pallichal local expressed only four isoforms with R_f values 0.07, 0.11, 0.2 and 0.25 (Table 10, Plate 11). Both genotypes exhibited same banding pattern. The PPO isozymes were expressed more prominently in the genotype Co-6 when compared to other genotypes analysed.

4.2.8 Qualitative determination of free amino acids

The thin layer chromatography was performed to determine the qualitative property of free amino acids. Five standard amino acids were run against the sample extracted from healthy and diseased cowpea plants. There was no significant difference between healthy and diseased samples except for their increased activity in healthy plants (Plate 12). The amino acid proline was more expressed in healthy when compared to the disease sample. The amino acid tyrosine was identified based on the relative mobility and was more expressed in the diseased sample. In addition to this, phenylalanine was also detected in healthy and diseased sample based on the mobility coefficient but with very low concentration.

4.3 Immunodetection

DAC – ELISA

DAC-ELISA was conducted to identify the virus infecting cowpea. The experiment was performed using monoclonal antibodies (mAbs) viz., 10 G5 and 16 G5 specific to BlCMV and mAbs specific to CABMV viz., 5H5, 7D9 and 6C10. The results of the experiment (Table 11) revealed that the mAb 10 G5 specific to BlCMV alone gave high reactivity towards the virus

Table 11 Reaction of virus in direct antigen coating ELISA

	Monoclonal antibodies	Absorbance at 405
Healthy sample	10 G5	0.134
Healthy sample	5 H5	0.106
BICMV	10 G5	2.03
BICMV	16 G5	0.00
CABMV	5 H5	2.06
CABMV	7 D9	2.09
CABMV	6 C10	2.19

Plate 11 Isozyme banding pattern of parents used in hybridization

Native gel electrophoresis of polyphenol oxidase isozyme

a. Sharika

b. Co-6

c. Pallichal local

d. Malika

S
S
C
C

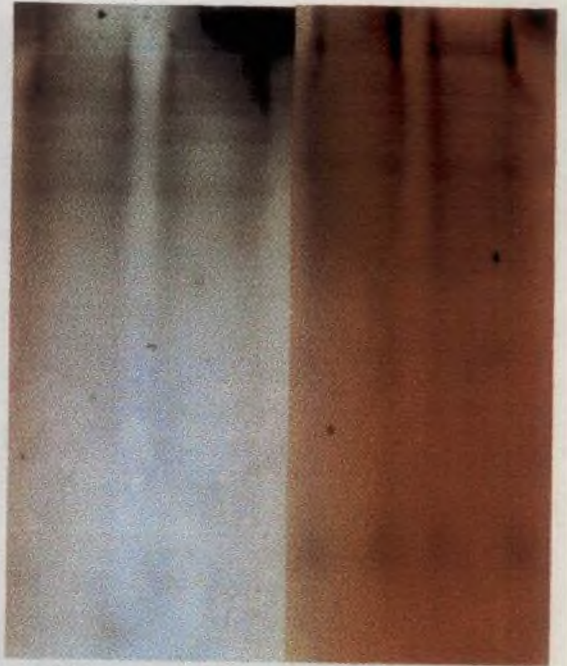
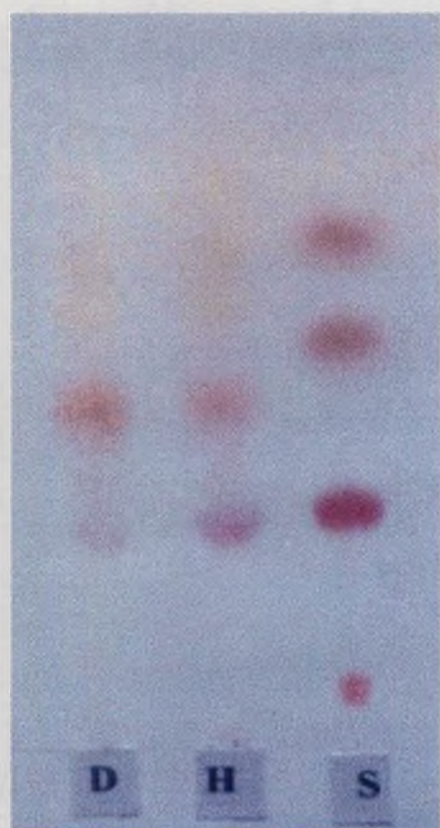

P
P
M
M

Plate 12 Thin layer chromatography showing the separated amino acids of healthy and BlCMV infected cowpea

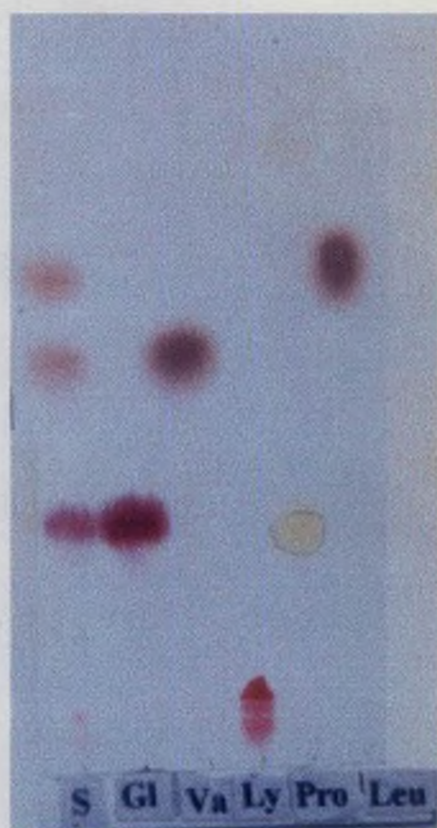
H – Healthy

D – Diseased

S – Standard amino acids



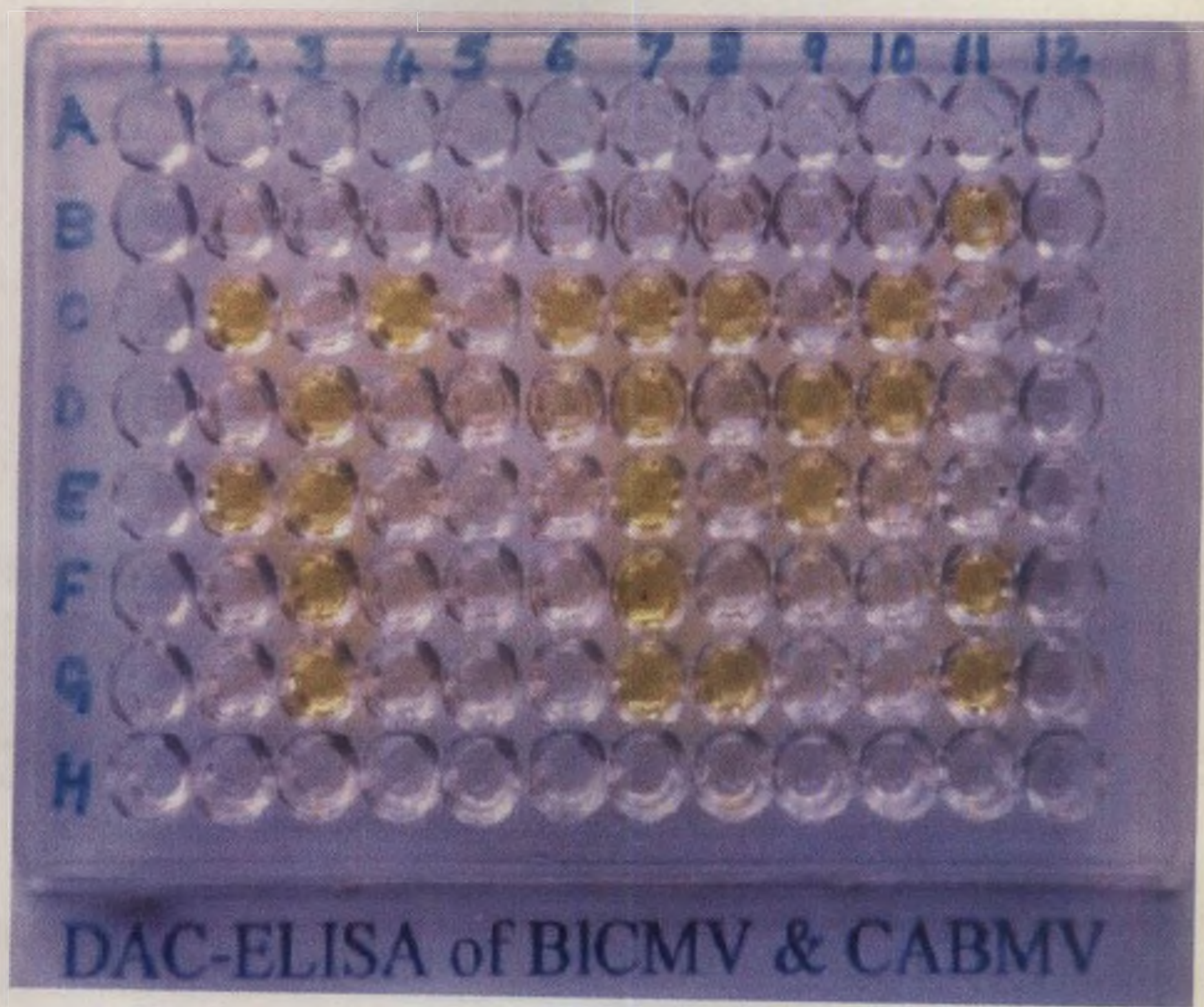
D
H
S



S
Gl
Va
Ly
Pro
Leu

Plate 13 Reaction of virus in DAC – ELISA

Well No.	Virus	Monoclonal antibody used
B11	CABMV	7D9
C2	BlCMV	10G5
C4	BlCMV	10G5
C6	CABMV	7D9
C7	CABMV	5H5
C8	CABMV	5H5
C10	CABMV	6C10
D3	CABMV	6C10
D7	CABMV	5H5
D9	CABMV	7D9
D10	CABMV	6C10
E2	CABMV	6C10
E3	CABMV	6C10
E7	BlCMV	10G5
E9	CABMV	5H5
F3	CABMV	5H5
F7	BlCMV	10G5
F11	CABMV	7D9
G3	CABMV	6C10
G7	CABMV	7D9
G8	CABMV	5H5
G11	CABMV	7D9



DAC-ELISA of BlCMV & CABMV

isolate, which gave an average absorbance of 2.03 at 405 nm. The monoclonal antibody 16 G5 specific to BlCMV gave negative reading. Monoclonal antibodies 5H5, 7 D9, 6 C10 also gave high reactivity (Plate 13). The average absorbance recorded by them were 2.06, 2.09 and 2.19 respectively at 405 nm indicating that the viruses infecting cowpea were BlCMV and CABMV.

Discussion

5. DISCUSSION

Cowpea (*Vigna unguiculata* (L.) Walp.) is an important vegetable crop cultivated through out Kerala. As previously reported, viral infections in legumes affect the biochemical and physiological performance of the plants. The magnitude of the induced effects depends on the particular virus and host plant resistance. Therefore, the present investigation was undertaken to analyse the effect of BlCMV infection on several physiological parameters related to host plant resistance.

Sixty six genotypes were screened against blackeye cowpea mosaic virus. Among the genotypes screened four were resistant, 29 medium resistant 27 medium susceptible and six susceptible. Co-6 which was found resistant to BlCMV was used as one of the parent to develop F_1 hybrids. The cultivated varieties Sharika, Malika and Pallichal local which were less tolerant to BlCMV was included in the present breeding programme to evolve high yielding and resistant varieties. Collins *et al.* (1985) inoculated 16 cowpea cultivars with major cowpea viruses and reported that Brown Crowder, Magnolia Blackeye, Missisippi Silver, Magnolia Purple and Worthmore had promising level of tolerance to BlCMV. Sreelakha (1987) reported the variety C-152 to be highly susceptible and variety CG-104 as tolerant to mosaic V-317 and V-276 was reported to be resistant to CABMV by Sudhakumari (1993). Kannan and Doraiswamy (1994) screened 50 cowpea varieties against BlCMV and reported that 30 were free from infection. Bashir and Hampton (1996) reported five genotypes (IT 8082049, Big Boy, Corona, Serido and Tennesse cream) immune to all seven isolates of BlCMV tested

and three genotypes TVu-2657, TVu-2740 and TVu-3435 were found immune to six isolates. Kline and Anderson (1997b) reported a BlCMV resistant variety UARK-M2. Gumedzoe *et al.* (1998) reported that germplasm accessions TVu 401, TVu 1453 and TVu 1948 and breeding lines IT 82D-885, IT 82D-889 and IT 82E-60 possess resistant genes to all six potyvirus isolates. Six cross combinations of four cultivars were carried out in the present investigation and F_1 hybrid seeds were collected for further evaluation.

Numerous sources of resistance to BlCMV were found in previous studies. With some of newly found sources of resistance to BlCMV in the present study, it may now be possible for the plant breeder to incorporate the virus resistance into cowpea varieties that have desirable horticultural characters.

The present study revealed that there was a reduction in the level of carbohydrate content in susceptible varieties inoculated with BlCMV compared to healthy control, the value decreased form 40.67 mg g^{-1} at one DAI to 24.33 mg g^{-1} at 15 DAI in case of susceptible variety, Sharika. Whereas it increased form 4.67 mg g^{-1} at one DAI to 8.67 mg g^{-1} at 15 DAI in case inoculated resistant variety. Such lower levels of carbohydrate in inoculated plants have been reported in cowpea by many workers (Ramaiah, 1978; Singh and Singh, 1984; Mayoral *et al.*, 1989; Umamaheswaran, 1996). Decreased photosynthesis and increased respiration occurred in virus infected tissues and lead to the altered concentration of carbohydrates (Bhavani *et al.*, 1998). Mali (2000) correlated that progressive increase in disease severity was associated with accumulation of total soluble carbohydrate and decrease

in starch content indicated the predominance of catabolic process after virus infection. Narayanaswamy and Ramakrishnan (1966) suggested that reduction in the level of carbohydrates, might be due to the breakdown of carbohydrate accelerated during respiration in virus infected plants. The results of the present studies also indicated that there was gradual depletion of total carbohydrate content in both compatible and incompatible host-virus interaction. The present study also agree with the findings of the earlier workers.

Virus infections are reported to cause reduction in chlorophyll in susceptible plants. The present study indicated that there was reduction in chlorophyll content in BICMV inoculated susceptible cowpea variety than the healthy control. There was no alteration in chlorophyll content in resistant cultivar Co-6 on inoculation. Similar cases of reduction in chlorophyll content in plants infected with cowpea mosaic virus has been reported (Ramaiah, 1978; Tripathi *et al.*, 1987; Mayoral *et al.*, 1989; Rao and Shukla, 1989). The present study also confirms the findings of earlier workers.

The present investigation revealed that following BICMV inoculation in susceptible cowpea varieties, there was an enhancement of total phenol content. It showed a peak value of 129.97 $\mu g\ g^{-1}$ at five DAI in Sharika. Ramaiah (1978) found that in variety Co-2 inoculated leaves had higher content of phenolics than that of healthy leaves. Ahmed *et al.* (1992) reported that after inoculation with yellow vein mosaic virus, total phenols, O-dihydroxyphenols and flavanols decreased in resistant lines. Similar results were obtained on present study also. Sutha *et al.* (1997) observed that the accumulation of phenolics in virus infected plants may be due to excess

production of hydrogen peroxide by increased respiration or due to activation of HMP shunt pathway, acetate pathway and release of bound phenolics by hydrolytic enzyme.

An increase in protein content was observed with increase in age of plant and also with BICMV inoculation. The value increased from 106 $\mu g\ g^{-1}$ at one DAI to 140.67 $\mu g\ g^{-1}$ at 15 DAI in susceptible variety. The protein content recorded a maximum concentration at 15 DAI in susceptible and resistant variety. Similar supporting evidences of enhanced level of protein content in virus infected plants have been reported by many authors (Singh and Singh, 1984; Singh and Singh, 1987; Yadav, 1988; Mayoral *et al.*, 1989 and Mali *et al.*, 2000). Increased protein content after inoculation, in both resistant and susceptible varieties and extend of increase more in susceptible lines than resistant have been reported by Ahmed *et al.* (1992). The drastic increase in total protein content after infection in susceptible cultivar was due to increase in viral proteins and or non-viral proteins occurring indirectly at the expense of normal host proteins directed by genes present in viral DNA.

It is evident from the present investigation that the accumulation of soluble proteins as observed in inoculated susceptible and resistant varieties agrees with the findings reported by many authors.

Investigation on changes in defence related enzymes *viz.*, peroxidase, polyphenol oxidase and phenyl alanine ammonialyase clearly indicated that there was significant increase in activities of these enzymes in inoculated plants. Peroxidase activity was found progressively increasing with age of plants. The peroxidase activity increased form 0.60 $min^{-1}\ g^{-1}$ to 3.07 $min^{-1}\ g^{-1}$

at 15 DAI in inoculated plants. Treatment of BICMV on resistant cultivar, Co-6 also found to accelerate the peroxidase activity which recorded a maximum of 5.47 min^{-1} g^{-1} at 15 DAI. Increase in peroxidase activity in susceptible cowpea varieties was reported by Khatri and Chenulu (1970), Wagih and Coutts (1982) and Radhika and Umamaheswaran (2000).

Polyphenol oxidase (PPO) activity increased in the resistant variety and reached a maximum of 0.16 min^{-1} g^{-1} at 10 DAI. Thereafter a sudden drop to 0.05 min^{-1} g^{-1} was observed at 15 DAI. Enhanced activity of PPO was also recorded in inoculated susceptible variety. Umamaheswaran (1996) reported that there was progressive increase in peroxidase, polyphenol oxidase and phenyl alanine ammonialyase activity in inoculated and susceptible varieties of cowpea.

PAL activity was also observed to be enhanced in case of inoculated susceptible plants when compared to healthy. The activity recorded a maximum value of 1827.62 nm g^{-1} min^{-1} at five DAI and then it dropped to 654.20 nm g^{-1} min^{-1} at 15 DAI. Zaidi *et al.* (1992) reported the changes in phenolic content and phenyl alanine ammonalyase in response to infection by carnation etche ring virus and the results strongly suggested the existence of a correlation between the elevated levels of phenolics and phenyl alanine ammonnialyase with disease resistance. The enhanced activity of oxidative enzymes at their peak activity oxidise phenolics to quinones, which in turn inactivate the virus infection, reported by Hampton and Fulton (1961) justify the enhanced activity in resistant cultivar to resist the virus infection.

Infection of plants with viruses is accompanied by the appearance of one or more new infection specific soluble protein. SDS-PAGE of soluble

proteins was analysed in cowpea following inoculation with BlCMV. Two separate experiments were performed to study the protein profile of healthy and diseased cowpea plants. The first experiment revealed that inoculation of cowpea with BlCMV did not result in the production of newly induced virus-related proteins, when compared to healthy control. The expression of protein in the diseased plants were more than in uninoculated control. In contrast samples analysed from plants at 15 DAI expressed the synthesis of two new virus related proteins in inoculated susceptible variety with R_f values 0.32 and 0.49 which was absent in uninoculated control. Protein synthesis in protoplast inoculated with cowpea mosaic virus (CPMV) nucleoprotein components was studied (Rottier *et al.*, 1980). Dumas (1988) reported that the increased synthesis of soluble protein was due to the hyper sensitive reaction of *Petunia* infected with TMV. Protein content estimated in the present investigation (Table 5) is also in agreement with this finding. The maximum content of 140.67 $\mu g\ g^{-1}$ was obtained at 15 DAI in susceptible plants. Cowpea mosaic virus infection gives rise to the formation of virus specific cytopathic structures which are presumed to have an essential function in virus replication (Hibi *et al.*, 1975). The synthesis of these structures 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 BlCMV.

The results presented in Plate 11 and Fig. 9 showed that there was distinct polymorphism in polyphenol oxidase isozymes of cowpea genotypes analysed. The two genotypes Co-6 and Pallichal local expressed four isoforms of PPO with R_f values of 0.07, 0.11, 0.20 and 0.25 indicating that

varieties are similar, whereas the susceptible varieties Malika and Sharika exhibited distinct polymorphism in isoPPO's between varieties. One apparently novel isozyme was detected at R_f 0.47 for Sharika and R_f 0.55 for Malika (Fig. 9). These polymorphisms are useful as genetic markers. As reported by Asiedu (1992) that isozyme analysis are powerful technique for identifying cultivars and germplasm accessions. The resulting polymorphism in the present investigation can also be used as genetic markers to identify resistant and susceptible genotypes.

Thin layer chromatography conducted to determine the qualitative property of amino acids showed that there was no significant difference between healthy and diseased samples except for their increased activity in healthy plants. The amino acid proline was more prominent in healthy, whereas the amino acid tyrosine was more expressed in diseased sample. Presence of phenylalanine was also detected in both healthy and diseased sample in low concentration. Qualitative and quantitative changes in free amino acids have been reported (Diener, 1960; Narayanaswamy and Ramakrishnan, 1966; Daniel, 1968 and Balakrishnan Nair, 1969). Miczynski (1959) found a lesser concentration of amino acids in diseased leaves than in healthy ones during the rapid spread of potato virus X in tobacco. On establishment of the disease there was an increase in concentration of amino acid in older leaves of inoculated plants. He observed an increase in aspartic acid and leucine and decrease in glutamic acid on diseased leaves. In present study, it was found that amino acid proline was expressed more in healthy and tyrosine in diseased sample. But this was contradictory to the report of John (1963) in which the expression of proline was more in tobacco leaf

infected with TMV. Only very limited number of amino acids were selected for the present study. A detailed study of amino acid analysis have to be undertaken to identify the specific amino acid involved during virus infection process.

A correct identification of the viruses infecting a crop is considered essential before adequate control measures can generally be developed. Many workers have developed simple and sensitive serological methods for the detection of virus in infected plants and vectors involved in their spread.

Direct antigen coating-Enzyme Linked Immunosorbent Assay (DAC-ELISA) was conducted using monoclonal antibodies specific to BlCMV and CABMV received from International Institute of Tropical Agriculture, Ibadan, Nigeria. DAC-ELISA was conducted to identify the virus in the plant samples from glasshouse maintained culture and from field. Results revealed that mAb 10G5 specific to BlCMV gives a high reactivity towards the virus isolate (Table 11). Monoclonal antibodies specific to CABMV also gave high reactivity indicating that the virus infecting cowpea were BlCMV and CABMV. Dijkstra *et al.* (1987) used ELISA to differentiate BlCMV and CABMV isolates from yard long bean. Huguenot *et al.* (1992) conducted ELISA and confirmed that BlCMV and CABMV are two different potyviruses. Gumedzoe *et al.* (1997) used serological test (ACP and DAS ELISA) with biotin, labelled monoclonal and polycolonal antibodies to identify BlCMV and CABMV serotypes of both potyvirus.

Radhika and Umamaheswaran (2000) found that the virus causing mosaic disease in cowpea was identified as blackeye cowpea mosaic virus using DAS and DAC ELISA. The test plants from glasshouse and field

showed reaction to both BlCMV and CABMV indicating that serologically, the isolates contained both BlCMV and CABMV which might be due to the combined infection of the virus. This result arise doubts about the existence of BlCMV and CABMV in the cultivated varieties of cowpea, eventhough these two viruses are two distinct viruses of potyvirus group reported by Huguenot *et al.* (1992). Serological differences however, should be regarded with caution, especially with potyviruses since antigenic determinants may differ depending on the way the virus has been purified or stored (Lima *et al.*, 1979).

Summary

6. SUMMARY

Studies were conducted on biochemical basis of disease resistance against blackeye cowpea mosaic virus causing severe mosaic disease in cowpea.

Sixty six varieties were screened for resistance to BlCMV under glasshouse condition. Four varieties were grouped as resistant, 29 moderately resistant, 27 moderately susceptible and six susceptible. The resistant variety Co-6 was crossed with other cultivated variety *viz.*, Sharika, Malika and Pallichal local and hybrid seeds were collected for evaluation.

Biochemical changes of host pathogen interaction was investigated. The carbohydrate content in leaves of resistant variety (Co-6) was lower than that of susceptible variety (Sharika). Following inoculation the carbohydrate content was significantly reduced in susceptible variety. The total chlorophyll content in BlCMV infected cowpea plants was found to be lower than uninoculated plants. There was a progressive increase in total phenol content of healthy plants with increase in plant age in susceptible variety. A lower level of phenol was found in resistant variety than in susceptible variety. The total soluble protein content was found higher in inoculated susceptible plants. There was a progressive increase in total soluble protein in both varieties in inoculated and uninoculated control plants. Analysis of defence related enzymes indicated that there was a progressive increase in peroxidase activity in healthy and inoculated plants of both susceptible and resistant varieties tested. Polyphenol oxidase activity was also found higher in case of resistant variety than in susceptible variety. In susceptible variety it was

enhanced following inoculation. Phenyl alanine ammonialyase activity was enhanced in case of inoculated susceptible plants when compared to healthy and maximum value was observed at five DAI. There was no significant difference in PAL activity in inoculated and uninoculated plants of resistant variety Co-6.

Sodium dodecyl sulphate–Polyacrylamide gel electrophoresis (SDS-PAGE) was carried out to analyse the protein profile of cowpea plants under inoculated and uninoculated conditions to investigate the presence of virus related proteins. The first experiment revealed that inoculation of cowpea with BICMV did not result in the production of newly induced virus-related proteins, compared to healthy control. The expression of protein in the diseased plants were more than that in uninoculated control. In contrast, samples analysed from plants at 15 DAI expressed the synthesis of two new virus related proteins in inoculated susceptible variety with R_f values 0.32 and 0.49 which was absent in uninoculated control.

Native polyacrylamide gel electrophoresis was performed for polyphenol oxidase isozyme. There was significant difference between genotypes analysed. Five isoforms of PPO were found in susceptible varieties Sharika and Malika, whereas other two genotypes Co-6 and Pallichal local expressed only four isoforms. The isoPPOs in Co-6 were more prominent compared to the other genotypes analysed.

Thin layer chromatographic separation of free amino acids revealed that there was no significant difference between healthy and diseased samples except for the increased expression of proline in healthy and tyrosine in diseased sample.

Immunodetection studies of virus infecting cowpea from field and glasshouse conditions revealed the presence of both BlCMV and CABMV. This indicated that there was combined infection of both the viruses in the susceptible varieties of cowpea.

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Appendix

APPENDIX – I

1) 0.01M Phosphate buffer (pH 7)

Stock solutions

Solution A : 1.36 g of KH_2PO_4 in 1000 ml

Solution B : 1.78 g of Na_2 HPO_4. $2H_2O$ in 1000 ml

51 ml of solution B and 49 ml of solution A are mixed and this gives a 0.01M phosphate buffer with pH 7.0.

2) 0.1M Phosphate buffer (pH 7.2)

A : 0.2M solution of monobasic sodium phosphate (27.8 g in 1000 ml).

B : 0.2M solution of dibasic sodium phosphate

(53.65 g of NaH_2 PO_4. $7H_2O$ or 71.7 g of Na_2 HPO_4. $12H_2O$ in 1000 ml).

39.0 ml of A is mixed with 61.0 ml of B diluted to a total of 200 ml.

3) 0.1M Sodium acetate buffer (pH 4.7)

Solution A : 0.2M of acetic acid (11.55 ml in 1000 ml)

Solution B : 0.2M of sodium acelate (16.4 g in 1000 ml)

23 ml of A and 27 ml of B are added together and diluted to 100 ml.

4) 0.1M Sodium phosphate buffer (pH 6.5)

Sodium A : 3.12 g of Na HPO_4 in 100 ml

Solution B : 3.55 g of Na_2 HPO_4 in 100 ml

68.5 ml of A added to 31.5 ml of B and made up to 200 ml

5) 0.1M Sodium borate buffer (pH 8.8)

A : 0.2M solution of boric acid (12.4 g in 1000 ml)

B : 0.05M solution of borax (19.05 g in 1000 ml)

50 ml of A is mixed with 30 ml of B and diluted to a total of 200 ml.

BIOCHEMICAL BASIS OF RESISTANCE AGAINST BLACKEYE COWPEA MOSAIC VIRUS IN COWPEA (*Vigna unguiculata* (L.) Walp.)

BY

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ABSTRACT OF THE THESIS
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ABSTRACT

The study was undertaken on the blackeye cowpea mosaic virus (BlCMV) causing a severe mosaic disease on cowpea (*Vigna unguiculata* (L.) Walp). It aimed at locating the sources of heritable resistance and biochemical basis of disease resistance required for formulating effective management practices to check the spread of the disease as well as immunodetection. Among the 66 varieties screened, four were resistant, 29 moderately resistant, 27 moderately susceptible and six susceptible. Six cross combinations were tried and F_1 hybrid seeds were collected for further evaluation. Biochemical changes indicated a lower carbohydrate content in the resistant variety, compared to susceptible variety. Chlorophyll content decreased in susceptible variety due to virus infection. A lower level of phenol content was observed in resistant variety. Increase in protein content was observed in both susceptible and resistant varieties upon inoculation. The defence related enzymes peroxidase, polyphenol oxidase and phenylalanine ammonialyase were enhanced with virus inoculation in susceptible variety. SDS-PAGE analysis of proteins with samples extracted from plants at 15 DAI showed the presence of two novel virus induced proteins in diseased samples. Native polyacrylamide gel electrophoresis performed for polyphenol oxidase isozyme revealed significant difference between the genotypes analysed. Five isoforms were found for Sharika and Malika and four for Co-6 and Pallichal local. There was no difference in amino acid pattern in healthy and diseased plant

samples in TLC, except for an increased expression of proline in healthy and tyrosine in diseased sample. Immunodetection could help identifying the viruses infecting cowpea as BlCMV and CABMV using specific monoclonal antibodies.